SEP 28 1936
MEDICAL LIBRARY

VOLUME 6 SEPTEMBER, 1936 NUMBER 5

American Journal of Clinical Pathology

OFFICIAL PUBLICATION
THE AMERICAN SOCIETY OF CLINICAL PATHOLOGISTS

CONTENTS

The Circulating Blood Cells as Seen by Darkground Illumination. O. C. HANSEN-PRUSS. . . . 423
A Study of Certain Factors which Influence the Sedimentation Rates of Erythrocytes with Especial Emphasis upon the Effect of Temperature. K. T. SASANO, W. H. ORDWAY AND E. M. MEDLAR. . . . 432
Use of the Stab Culture in the Growth of Certain Pathogenic Fungi. JOHN W. WILLIAMS. . . . 444
Borderline Silicosis. HENRY C. SWEANY. . . . 448
Studies of Urobilinogen. I. An Improved Method for the Quantitative Estimation of Urobilinogen in Urine and Feces. CECIL JAMES WATSON. . . . 458
A Study of the Organisms and Serums from a Number of Typhoid Patients and Carriers. LOIS ALMON AND W. D. STOVALL. . . . 476
Erythrocyte Counts, Hemoglobin, and Erythrocyte Volume in Normal Young Men and Women Residing in the Eastern United States. WM. P. BELK, ELSIE CURTIS AND MARGARET K. WILSON. . . . 487
The Use of Calculation Charts for the Wintrobe Hematocrit and Sedimentation Rates. F. LOWELL DUNN AND JOHN C. SHARPE 497
A Simple Slide Test for Syphilis. SEABORN J. LEWIS. . . . 502
Editorial. . . . 507
News and Notices. . . . 514
Book Reviews. . . . 516

PUBLISHED BI-MONTHLY BY THE WILLIAMS & WILKINS COMPANY
MOUNT ROYAL AND GUILFORD AVES., BALTIMORE, U. S. A.

Made in United States of America

American Journal of Clinical Pathology

EDITOR

T. B. MAGATH, Mayo Clinic, Rochester, Minnesota

ADVISORY EDITORIAL BOARD

C. S. BUTLER, U. S. Navy Medical Supply Depot, Brooklyn, N. Y.

H. J. CORPER, National Jewish Hospital, Denver, Colo.

B. C. CROWELL, American College of Surgeons, Chicago, Ill.

HERBERT FOX, Pepper Laboratory of Clinical Medicine, University of Pennsylvania, Phila., Pa.

A. S. GIORDANO, 531 North Main Street, South Bend, Indiana.

F. W. HARTMAN, Henry Ford Hospital, Detroit, Mich.

R. A. KILDUFFE, Atlantic City Hospital, Atlantic City, N. J.

J. A. KOLMER, Temple University School of Medicine, Philadelphia, Pa.

S. P. REIMANN, Lankenau Hospital, Phila., Pa.

A. H. SANFORD, Mayo Clinic, Rochester, Minnesota

WALTER M. SIMPSON, Miami Valley Hospital, Dayton, Ohio

W. S. THOMAS, Clifton Springs Sanitarium and Clinic, Clifton Springs, N. Y.

WARREN T. VAUGHAN, 808 Professional Bldg., Richmond, Va.

American Society of Clinical Pathologists

OFFICERS

President, ROY R. KRACKE, Emory University, Emory University, Georgia

President-elect, C. W. MAYNARD, Pueblo Clinic, Pueblo, Colorado

Vice-President, FREDERICK C. NARR, Research Hospital, Kansas City, Missouri

Secretary-Treasurer, A. S. GIORDANO, 531 North Main Street, South Bend, Indiana

STANDING COMMITTEES

EXECUTIVE COMMITTEE

F. H. LAMB, Chairman, Davenport, Iowa
W. M. SIMPSON, Dayton, Ohio
R. A. KILDUFFE, Atlantic City, New Jersey
L. W. LARSON, Bismarck, North Dakota
A. G. FOORD, Pasadena, California
K. IKEDA, St. Paul, Minnesota

BOARD OF CENSORS

A. V. ST. GEORGE, Chairman, New York, New York
C. G. CULBERTSON, Indianapolis, Indiana
O. W. LOHR, Saginaw, Michigan
I. A. NELSON, Tulsa, Oklahoma
S. P. REIMANN, Philadelphia, Pennsylvania
H. A. HEISE, Milwaukee, Wisconsin

BOARD OF REGISTRY OF TECHNICIANS

PHILIP HILLKOWITZ, Chairman, Denver, Colorado
R. R. KRACKE, Emory University, Georgia
H. H. FOSKETT, Portland, Oregon
K. IKEDA, St. Paul, Minnesota
I. DAVIDSOHN, Chicago, Illinois
ASHER YAGUDA, Newark, New Jersey

Entered as second-class matter, January 7, 1931, at the Post Office at Baltimore, Md., under the act of March 3, 1879
Copyright 1936, The Williams & Wilkins Company

THE CIRCULATING BLOOD CELLS AS SEEN BY DARKGROUND ILLUMINATION

O. C. HANSEN-PRUSS

Department of Medicine, School of Medicine, Duke University, Durham, North Carolina

The darkfield microscope has been used extensively in hematology,* but the advantages of its routine use have not been sufficiently emphasized. It is the purpose of this paper to point out the simplicity and reliability of darkfield studies of the cells of the circulating blood, to describe briefly the appearance and different characteristics of such cells, and to urge that the method be more widely used both in the teaching of students and by practitioners of medicine.

METHOD

A drop of blood is obtained on a coverslip, placed on a slide and sealed with vaseline or paraffin. Perfectly clean glassware is essential. The erythrocytes, in a satisfactory preparation, are evenly distributed, without rouleau formation or clumping. Unless the room is well heated, the slide should be kept in a warm box at 37°C. for 20 or 30 minutes. After this the warmth from the illumination is sufficient, and may prove excessive. The granular leukocytes should show active amoeboid movement after 5 to 10 minutes at 37°C. Such darkfield preparations, if the warmth is properly controlled, remain satisfactory for at least 4 hours. They may be compared with supravitally stained, moist preparations, using neutral red or Janus green, the two methods of study nicely supplementing each other.

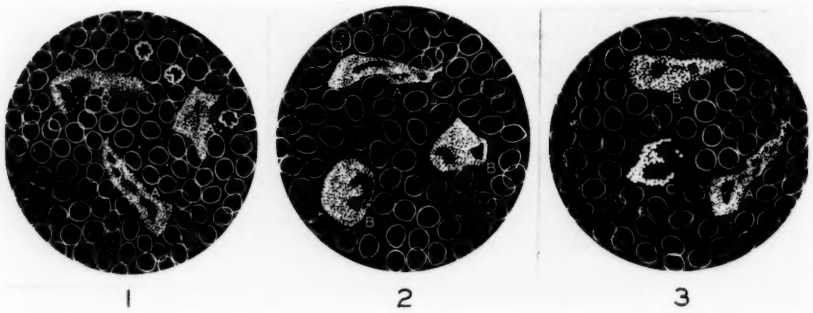
The characteristics of the nine formed elements of the circulating blood are as follows:

ADULT MYELOID LEUKOCYTES

The three types of leukocytes (figs. 1, 2, 3), neutrophiles, eosinophiles, and basophiles may be clearly differentiated by the size

* A selected list of helpful papers in this field is appended.

and refractility of the granulations, and by differences of motility. The granules of the neutrophiles are small, of even size and exhibit a homogeneous, silvery refractivity. The eosinophiles are closely packed with larger granules which have a slightly golden sheen. In the basophilic cells the granulations are quite coarse and do not appear to be of even consistency; the center of the granules being often devoid of refractile material, giving them a hollow appearance. This phenomenon is seen occasionally in eosinophilic granules, and in this case the number of granules and the degree of motility will furnish differential criteria. The nuclei and cell membranes of leucocytes are not refractile.



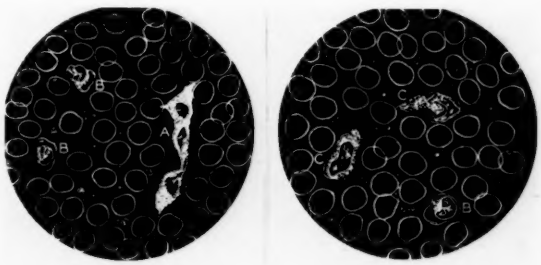
FIGS. 1, 2, 3. CAMERA LUCIDA DRAWINGS OF FRESH (HUMAN) BLOOD PREPARATIONS. The magnification is approximately 1800×. They show adult myeloid granulocytes in various phases of motion. *A* = Neutrophilic leukocytes. *B* = Eosinophilic leukocytes. *C* = Basophilic leukocytes.

The three types of leukocytes vary markedly in motility. Amoeboid movements are most active in the neutrophiles and least in the basophiles. The degree of motility in the eosinophiles is markedly less than in the neutrophiles. The granules in all three types show a scintillating, Brownian-like type of non-progressive movement.

LYMPHOCYTES

Adult lymphocytes (figs. 4, 5) are very easily recognized. These cells have a fairly distinct, dully refractile pericellular membrane. In some, this membrane is slightly undulating, par-

ticularly in the large lymphoid cells which are endowed with a sluggish amoeboid motility. Bud-like projections represent the most common type of motility seen in the adult lymphocyte. The cytoplasm is non-refractile. In many instances, one can detect a poorly (grayish) refractile zone immediately about the nucleus. This zone is not strictly homogeneous but seems to be due to the presence of semi-granular material. Many of the large lymphocytes contain highly refractile granules in the cytoplasm; these granules vary in size and are quite stationary within the cell. They may be in the non-refractile or in the dully refractile portion of the cytoplasm. The nucleus is sharply outlined



4 5

FIGS. 4, 5. CAMERA LUCIDA DRAWINGS OF FRESH (HUMAN) BLOOD. Magnification approximately 1800×. *A* = Neutrophilic leukocytes. *B* = Lymphocytes. *C* = Monocytes.

by a fairly thick, dully refractile membrane. The structure of the nucleus of lymphocytes varies from that of adult myeloid cells in that it contains material which is definitely refractile.

MONOCYTES

The monocytes present a characteristic appearance in the dark-field. It is well known that these macrophages have a variable degree of motility. They have a fairly well-defined pericellular membrane, which is poorly refractile. The pericellular membrane of the monocyte is less distinct than that of the lymphocyte. The cytoplasm proper is non-refractile. Within the cytoplasm one can always see granulations which vary in number, size and degree

of refractility. These granulations frequently float about within the cytoplasm. If the cell assumes progressive motion, the granulations follow the cytoplasmic protrusions. In numerous monocytes, one can see, in addition, a few to many large, highly refractile globules. These often increase in size and as the cell grows older, seem to float about sluggishly within the cytoplasm. After a simultaneous study of preparations stained supravitally with neutral red and Janus green, I believe that these refractile, globule-like granules represent the so-called segregation granules.

The nucleus of the monocyte is well defined by darkfield illumination. The shape of the nucleus varies; it may be round, ovoid, elongated or sickle-shaped. The perinuclear membrane is thinner, and apparently, more delicate than that surrounding the nucleus of the lymphocytes, in some instances it may not be seen. The whole nucleus is often dimly refractile, but in every cell some amount of nuclear substance can be seen. Scattered throughout the nucleus there are, frequently, rather dense masses which are poorly refractile. They correspond to the chromatic masses which are usually seen in the nucleus of monocytes stained with one of the Romanowsky dyes.

IMMATURE MYELOID LEUKOCYTES

The myeloblast (figs. 6, 7, 8) has a well-defined, fairly refractive pericellular membrane, but the cytoplasm is non-refractile and the cell non-motile. The nucleus is round and is enclosed in a sharply defined membrane of grayish refractility. Within the nucleus there are strands of grayish chromatin. Two or more faintly refractile structures can usually be seen imbedded in the chromatin network; these undoubtedly represent nucleoli. As the cell grows older, there appears about the nucleus a zone of increasing refractility, which is homogeneous and surrounds the perinuclear membrane rather lightly. I have been unable to see definite mitochondria in unstained specimens. It is possible that they are imbedded in this perinuclear zone. In the somewhat older myeloblasts the perinuclear matter begins to break up into ill-defined masses. While this process is going on, strands of refractile material extend to the periphery of the cell which is soon

outlined by a ring of grayish-refractile, amorphous material. It is in this zone that the first definite granulations appear. These granulations are at first somewhat larger than those seen in the adult leukocytes. As the granulations increase in number they become smaller and more refractile, and the previously homogeneous zone of grayish and dull refractility gradually vanishes. Coincidentally, the nucleoli disappear and the nuclear chromatin becomes less refractile. Very soon after the granulations form, the perinuclear membrane begins to lose its refractility. Shortly after the first definite granulations have appeared, a gradually

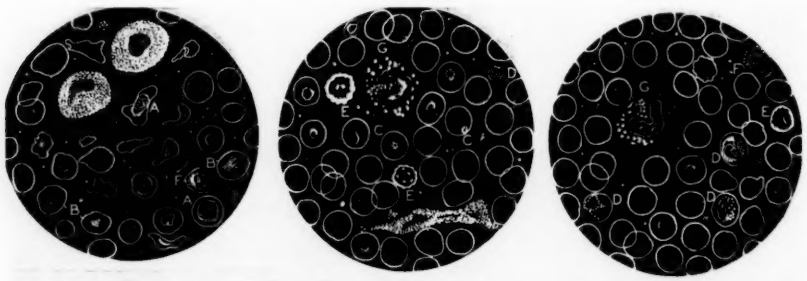
FIGS. 6, 7, 8. CAMERA LUCIDA DRAWINGS OF FRESH (HUMAN) BLOOD PREPARATIONS. The magnification is approximately 1800×. *A*, *B* = Myeloblasts. *C*, *D* = Pre- or undifferentiated myelocytes. *E* = Neutrophilic myelocyte. *F* = Eosinophilic myelocyte. *G* = Basophilic myelocyte. *H* = Lymphoblast. *I*, *J* = Lymphoid cells. *K* = Monocyte. *N* = Nucleated erythrocyte.

increasing number of these granules begin to move to and fro in a restricted space, only the highly refractile granules showing this motion. It can best be described as "scintillation." As more granules appear, this motion spreads from the periphery to the center of the cell, due apparently to increasing fluidity of the cytoplasm. The refractility of the granulations also increases as the cell grows older, and spreads from the periphery to the center.

LYMPHOBLASTS, MONOBLASTS

Lymphoblasts in the darkfield do not present very striking structural characteristics. Their appearance is very similar to

that of older lymphoid cells. The cytoplasm of the young lymphoid cells seems denser and consequently it can frequently be visualized as a homogeneous mass of faint, grayish refractility. This is not true of the cytoplasm of the myeloblast. The nucleus of the lymphoblast is often quite large and its chromatin is distinctly refractile. Structures resembling nucleoli can be seen as a rule. The refractility of the perinuclear and pericellular membranes is greater in the young lymphoid cells than in adult lymphocytes. In some lymphoblasts a round mass of fine, fairly refractile and non-motile granules lie very close to the nucleus.



FIGS. 9, 10, 11. CAMERA LUCIDA DRAWINGS OF FRESH, UNSTAINED HUMAN BLOOD SPECIMENS. Magnification approximately 1800×. Fig. 9 is from a case of erythroblastic anaemia. Figs. 10 and 11 are from patients infected with *P. vivax*. *A* = Erythroblast. *B* = Erythrocyte containing nuclear chromatin. *C* = Young trophozoites. *D* = Amoeboid trophozoites. *E* = Crenated erythrocytes. *F* = Lymphocyte. *G* = Monocyte.

Monoblasts may be distinguished from the older monocytes by the possession of thicker and more refractile perinuclear and pericellular membranes, and by absence of cytoplasmic granulations. The nuclear chromatin is not quite so refractile as that of the lymphoblasts, but more so than that of the myeloblasts. Likewise the compactness and refractility of the cytoplasm of the monocyte becomes less as the cell grows older.

ERYTHROCYTES

The pericellular membrane of the erythrocyte is quite definite and rather highly refractile. As the cell deteriorates, this membrane becomes thicker, and knob-like protrusions and indentations

of a yellow or golden color appear. The hemoglobin is non-refractile (figs. 9, 10, 11).

MEGALOBLASTS

The megaloblasts contain no hemoglobin, yet the cell cytoplasm remains opaque except for a narrow zone about the nucleus. This zone is unusually granular and a has silvery sheen. The perinuclear membrane is distinct. Nucleoli can not be seen. In the erythroblasts, the perinuclear refractile mass is barely seen. In the older cells, the perinuclear membrane and the nuclear chromatin becomes increasingly less refractile. The normoblast is distinguished from the mature red cell only by the presence of a nucleus defined by a faintly perceptible perinuclear membrane. In blood specimens from erythroblastic anaemia, and in others containing a demonstrably large number of reticulocytes by vital staining, the darkfield reveals erythrocytes which contain a silvery mass of refractile particles, sometimes arranged in a delicate network. It is possible that such cells correspond to reticulocytes. I have classified them as "red cells with remnants of chromatin," because counts of such cells do not correspond to simultaneous reticulocyte estimations by the brilliant cresyl blue method. Fresh blood stained supravitally for reticulocytes can be examined in the darkfield; the reticulocytes stand out strikingly with a greenish, golden, sometimes bluish refulgence.

PLATELETS

Blood platelets are easily seen. The refractility of these elements varies, but is usually quite marked. Structural details cannot be distinguished, and they appear as more or less homogeneous masses. In every preparation there are also numerous other very small masses, which are fully refractile. These float about in the specimen and increase in number as it grows older. This is the so-called "blood dust." In my experience, blood dust is most abundant in hemolytic anaemias.

PLASMODIA

Malaria parasites are recognized with beautiful clarity in the darkfield. Extensive experience is not required. The pigment is

highly refractile, and identifies the organism. In the older parasites the protoplasm also becomes somewhat refractile. Many of the plasmodia remain viable and motile for 10 to 16 hours in such preparations, and it is possible to observe their growth and development. The very young, pigment-less trophocyte (age, 1 to 5 hours) are usually not seen by darkground illumination. Crenation and the changes due to uneven distribution of the hemoglobin can produce refractile areas in the erythrocyte, which may be confused with malaria parasites. As soon as the pigment appears and the parasite is old enough to acquire rather marked motility (age, 6 hours), it is easily seen and can hardly be confused with artefacts. The pigment is very brightly refractile and silvery. The protoplasm of the older parasite (age, 12 hours) is faintly refractile; this is of help in estimating the age of the organism. *P. falciparum* gametocytes resemble golden crescents studded with silver granules. Gametocytes of the *P. malaria* and *P. vivax* have no special characteristics in the darkfield and cannot be distinguished from the older amoeboid forms, due to the non-refractility of the malaria chromatin.

CONCLUSIONS

The study of human blood cells by the darkfield method is a very simple and reliable technical procedure. It is easily possible to identify and minutely analyze the various characteristics of these cells. Malaria parasites are seen with particular ease. The method avoids the involved and somewhat unreliable technical factors encountered in the use of the supravital dyes and in staining of fixed smears.

REFERENCES

(1) Brugsch, Th., and Schilling, V.: Die Kernform der lebenden neutrophilen Leukozyten beim Menschen. Folia haematol., **6:** 327–336. 1908.

(2) Dietrich, A.: Rote Blutkörperchen bei Dunkelfeldbeleuchtung. Verhandl. d. deutsch. path. Gesellsch., **12:** 202–209. 1908.

(3) Guaidukov, N.: Dunkelfeldbeleuchtung und Ultramikroskopie in der Biologie und in der Medezin. Jena: G. Fischer, 1910.

(4) Kopac, M. J.: Darkfield micromanipulation with an Ultropaque illuminator. Science, **82:** 70. 1935.

(5) LEWIS, M. R.: Reversible gelation in living cells. Bull. Johns Hopkins Hosp., **34:** 373–379. 1923.

(6) LEWIS, W. H.: Observations on cells in tissue-cultures with darkfield illumination. Anat. Rec., **26:** 15–29. 1926.

(7) SCHILLING, V.: Lebende weisse Blutkörperchen im Dunkelfeld. Folia haematol., **6:** 429–443. 1908.

(8) SIEKE, F.: Blutkörperchenzahlung im Dunkefeld. München med. Wchnschr., **74:** 891. 1927.

(9) STRANGEWAYS, T. S. P., AND CANTI, R. G.: The living cell in vitro as shown by dark-ground illumination and the changes induced in such cells by fixing reagents. Quart. Jour. Micr. Sc., **71:** 1–14. 1927.

(10) STÜBEL, H.: Ultramikroskopische Studien über Blutgerinnung and Thrombocyten. Pflüger's Arch., **156:** 361–400. 1914.

(11) TAKEUCHI, K.: Untersuchungen über Blutfäden. Folia haematol., **34:** 259–280. 1927.

A STUDY OF CERTAIN FACTORS WHICH INFLUENCE THE SEDIMENTATION RATES OF ERYTHROCYTES WITH ESPECIAL EMPHASIS UPON THE EFFECT OF TEMPERATURE

K. T. SASANO, W. H. ORDWAY AND E. M. MEDLAR

From the Hegeman Memorial Research Laboratory of the Metropolitan Life Insurance Company Sanatorium, Mt. McGregor, New York

The increasing use of the sedimentation rate of erythrocytes as indicative of a pathological state suggests that clinicians are finding the information gained from this simple test of sufficient value to warrant its inclusion in the list of routine laboratory procedures. Since the original reports of Fahraeus[3] and Westergren [13], many modifications of technic have been reported. These modifications have had largely to do with the diameter and length of the tubes, the type of anticoagulant, and the amount of blood used. Cutler[1] devised a graphic method, and later Cutler[2] and Morriss and Rubin[7] suggested the use of micro-methods. Walton[12] and others advocated a standardization of the test on a basis of the number of erythrocytes per cubic millimeter. Wintrobe[14] reported that using a hematocrit, the sedimentation rate, the cell volume and a rough estimate of leukocytes and platelets can all be obtained from a single sample of blood.

Few reports indicating the various factors which may influence the sedimentation rate have appeared in the literature. Maia[6] and Scala[10] stated that low temperatures tend to retard and higher temperatures to accelerate the sedimentation rate. Gordon and Cohn[4] also have found that temperature affects the rate of sedimentation and advocated a standard room temperature of 23°C. Velicogna[11] reported that altitude also has some effect on sedimentation rate.

This report deals with an investigation of the effect of temperature and altitude, manipulation of blood and anticoagulants on the rate of erythrocytic sedimentation, and a body temperature sedimentation technic.

Comparative sedimentation rates of 200 consecutive tests done in the summer and winter at room temperatures are given in table 1. The differences noted suggest that the higher temperatures in summer had some bearing on the higher sedimentation rates as compared to the average winter sedimentation rate.

TABLE 1

A Comparison of Winter and Summer Sedimentation Rates in One Hour

Average of 200 consecutive admission cases

Tubes	Winter	Summer	Ratio
	mm.	*mm.*	
Large	10	14	1:1.40
Small	7.3	10.7	1:1.46

TABLE 2

Effect of Temperature on Sedimentation Rates of Erythrocytes

Number	9°C.		21°C.		37.5°C.		Number	7°C.		18°C.		37.5°C.	
	1 hr.	2 hrs.	1 hr.	2 hrs.	1 hr.	2 hrs.		1 hr.	2 hrs.	1 hr.	2 hrs.	1 hr.	2 hrs.
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*		*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	3	5	7	17	15	23	11	2	4	5	13	10	21
2	0.5	1	3	7	4	8	12	2	4	4	18	7	15
3	11	23	24	40	35	43	13	2	4	5	9	8	17
4	18	35	38	50	48	52	14	3	5	7	15	14	25
5	1.5	2.5	3	6	4	9	15	11	27	33	48	50	56
6	2	3	4	9	8	18	16	2	5	8	18	19	28
7	2.5	5	4	13	13	21	17	1	2	1.5	3	3	8
8	3	7	7	16	14	19	18	5	12	22	33	34	41
9	3	6	9	18	16	28	19	19	31	35	52	50	61
10	1	2	2	4	3	7	20	10	24	54	57	57	58
Average of 20 cases								5.1	10.8	13.7	22.2	20.6	27.9

To ascertain the ultimate effects of temperature on sedimentation rates samples of the same specimen of blood were placed in comparable tubes at 6°–9°C.; 18°–27°C.; and 37.5°C. From table 2 it will be seen that there is a consistently greater rate of sedimentation at 37.5°C. This is especially brought out by the averages at the bottom of each column.

For purpose of determining the effect of barometric pressure,

TABLE 3

EFFECT OF BAROMETRIC PRESSURE ON SEDIMENTATION RATE OF ERYTHROCYTES

NUMBER	TEMPERATURE	BAROMETRIC PRESSURE			
		730 mm.		590 mm.	
		1 hr.	2 hrs.	1 hr.	2 hrs.
	°C.	mm.	mm.	mm.	mm.
1	18	18	32	20	35
2	18	20	37	24	40
3	18	12	27	14	31
4	18	20	34	21	34.5
5	18	5	10	5.5	11.5
6	18	14	27	15	29
Average.....		14.8	27.8	16.6	30.1
		743 mm.		560 mm.	
		mm.	mm.	mm.	mm.
7	23	14	27.5	15	28
8	23	2.5	7	3.5	10
9	23	18	33	21	36
10	23	11	23	13	26
Average.....		11.4	22.6	13.1	25

TABLE 4

SEDIMENTATION RATES OF BLOOD WITH DIFFERENT AMOUNT OF ERYTHROCYTES

CASES	RATE IN 1 HR.	CELL VOLUME	ERYTHROCYTES
	mm.	per cent	
1	10	51	5,340,000
2	7	58	6,100,000
3	50	49	4,620,000
4	7	50	4,700,000
5	24	40	4,120,000
6	4	39	3,260,000
7	9	40	3,800,000
8	34	41	4,300,000
9	23	42.5	4,180,000
10	11	42.5	4,200,000

identical samples of blood were placed in vacuum jars at pressures of 560, 590, 730 and 743 mm. of mercury at room temperature. The results given in table 3 show that while the barometric pres-

sure does affect the sedimentation rate, the difference is not significant.

TABLE 5

(A) The Effect of Normal and Pathological Plasma on Sedimentation Rate

CASE	GROUP	ERYTHROCYTES	MIXTURE	CELL VOLUME	HEMOGLOBIN	SEDIMENTATION RATE 1 HR.
			cc.	*per cent*	*grams*	*mm.*
1	O	4,270,000		41.5	12.6	30
2	O	4,510,000		44.5	14.2	6
1 (plasma) 2 (cells)	O O		0.555 0.445	44.5		28
2 (plasma) 1 (cells)	O O		0.585 0.415	41.5		9

(B) Interchanging Plasma and Cells of Normal and Pathological Blood

3	A	Original citrated blood		46.5		3
4	A	Original citrated blood		44.0		36
3 4	A A		Plasma Cells	45.0		6
4 3	A A		Plasma Cells	47.5		32
5	O	Original citrated blood		48.0		4
6	O	Original citrated blood		41.0		50
5 6	O O		Plasma Cells	42.0		10
6 5	O O		Plasma Cells	48.0		42

Note: Higher cell volume in mixed blood is due to the loss of plasma in transfer. Higher sedimentation rate in normal plasma with pathological cells, may be due to the presence of some pathological plasma carried over with the cells.

The effect on the sedimentation rate of decreasing the number of erythrocytes as reported by Walton[12] was carefully investigated and was corroborated in the main. The highly complex colloidal

nature of blood and the considerable manipulation of the blood necessary in performing this test offer a serious objection to this procedure. Walton's findings are of academic interest but it is doubtful whether they are of practical importance since the rate of sedimentation of erythrocytes is not always inversely proportional to the number of erythrocytes. In table 4 it will be seen that the rate of sedimentation of erythrocytes is frequently independent of the number of cells.

From these investigations it seems to us that the condition of the blood plasma is of far greater significance than the erythrocytes in the sedimentation rate. To demonstrate that the blood plasma has a greater bearing than the number of erythrocytes in altering the sedimentation rate, the following test was made. Two individuals, belonging to the same blood group and exhibiting grossly different sedimentation rates, were selected. One of the individuals was normal, the other ill clinically. The erythrocyte volume and sedimentation rate for each case was determined, and the plasma and erythrocytes were separated with as little manipulation as possible. Then the erythrocytes of one were mixed with the plasma of the other, after due allowances had been made for the differences in the cell volumes. It will be seen in table 5 that when the erythrocytes from the normal blood are mixed with the plasma of the pathological blood, there is an approximation of the original high sedimentation rate; while when the normal blood plasma is mixed with the erythrocytes from the high sedimentation case, there is very little change in the rate from the original reading.

It is generally recognized that the type of anticoagulant used influences the sedimentation rate[9]. Magath and Hurn[5], in a recent publication, stated that one cc. of 1.1 per cent sodium oxalate solution in 5 cc. of blood, though not isotonic to erythrocytes, gives results which correspond closely with those obtained by the use of heparin, in cell volume determination. We have found that both sodium and potassium oxalate used in the strength advocated by Magath and Hurn are very satisfactory in the sedimentation test also. If the results obtained by the use of heparin are generally accepted as the standard for cell

volume, then it appears that the use of 1.1 per cent solution of sodium or potassium oxalate is advisable if an anticoagulant

TABLE 6

(A) The Effect of Type of Anticoagulant on Sedimentation Rate

CASES (SAME DAY, SAME HOUR)	20 PER CENT OF 4 PER CENT SODIUM CITRATE SOLUTION	SOLID POTASSIUM OXALATE 0.08 PER CENT SOLID AMMONIUM OXALATE 0.12 PER CENT
	mm.	*mm.*
1	5	8
2	17	24
3	28	38
4	8	13
5	4	6
6	2	3
7	8	15
8	15	25
9	5	8
10	28	33
Average	11.2	17.3

(B) Comparative Sedimentation Rates and Cell Volumes

BLOOD	SEDIMENTATION RATES IN 1 HOUR		CELL VOLUMES		
	Isotonic citrate solution	1.1 per cent potassium oxalate solution	Citrate solution	Oxalate solution	Solid oxalate
	mm.	*mm.*	*per cent*	*per cent*	*per cent*
1	14	16	41.5	52	44.5
2	10	11	46.5	55.5	49
3	32	33	33.5	39.5	
4	16	17	36	42.5	
5	6	6	43	48	45
6	27	28	37	42	40.5
7	13	14	39.5	56.5	
8	6	6	36.5	44.5	39
9	21	21	41.5	48	47
10	11	11	44	52.5	
Average	15.6	16.3	40.9	47.8	44.1

other than heparin is used. An isotonic sodium citrate solution gives much lower cell volume. (See table 6.)

Wintrobe[15] advocated a room temperature of 22°–27°C. and

the use of solid potassium oxalate and ammonium oxalate (4 and 6 mgm. respectively for 5 cc. of blood). For cell volume determination, a solid anticoagulant is preferable although slight hemolysis may occur in some samples of blood. When a large number of tests are performed, we have found it more convenient to use an isotonic solution of anticoagulant, or 1.1 per cent potassium oxalate solution. Room temperatures over the country as a whole represent an extremely variable factor especially at different seasons of the year. We are of the opinion that it is preferable to have all sedimentation tests done at the normal body temperature (incubator at 37.5°C.) for two reasons: (1) the blood will then be at the temperature which approximates normal conditions, (2) a uniform temperature for all localities, regardless of seasonal variations, is obtained.

The size of the tube used in performing the sedimentation test also influences the rate (see table 1). During the past eleven years we have tried many methods and have come to the conclusion that a tube with an inside diameter of 6 mm. is the most satisfactory because the blood can be oxalated in the same tube. With the more frequent use of the sedimentation test and because of objections on the part of some patients to venipuncture, the finger tip method for obtaining blood was adopted. After careful checking against our standard method we found little variation in the result if the temperature factor remained constant. To obtain blood fairly easily, a rather deep puncture is made with a blood lancet. A tube 200 mm. in length graduated at 25 mm., 100 mm., and 125 mm. with an inside diameter of 2 mm. is used to collect the blood. A 1.1 per cent solution of sodium oxalate is drawn up to the 25 mm. mark; the blood is then collected by capillary attraction and gravity from the finger tip puncture up to the 125 mm. mark. The blood is then gently blown into a small test tube where it is thoroughly mixed by drawing the blood up and down several times. Finally this mixture is drawn up to 100 mm. and placed in a rack in a vertical position and put in the incubator at 37.5°C.

Of late we have adopted a modification of the Wintrobe[14] and Plass and Rourke[8] method. The oxalated blood is introduced

TABLE 7

Distribution of Sedimentation Rates in Different Groups

(*A*) *The sanatorium employees who have passed the physical examinations (380)*

Rates in 1 hour	Body temperature		Room temperature	
	Male	Female	Male	Female
	Number of individuals			
	100	100	100	80
mm.	*per cent*	*per cent*	*per cent*	*per cent*
0–2	10	2	30	2
2.1–5	51	9	54	34
5.1–8	19	24	8	31
8.1–11	5	29	8	13
11.1–14	8	20	0	8
14.1 and up	7	15	0	13

(*B*) *The non-tuberculous patients at admission (671 cases)*

	Number of individuals			
	250	150	157	114
mm.	*per cent*	*per cent*	*per cent*	*per cent*
0–2	9	4	23	4
2.1–5	17	6	37	31
5.1–8	15	15	15	27
8.1–11	16	15	5	12
11.1–14	11	15	5	6
14.1 and up	32	45	15	20

(*C*) *The tuberculous patients at admission (385 cases)*

	Number of individuals			
	63	86	126	110
mm.	*per cent*	*per cent*	*per cent*	*per cent*
0–2	2		14	8
2.1–5	5	3	28	21
5.1–8	10	7	16	14
8.1–11	8	10	10	11
11.1–14	11	7	5	9
14.1 and up	64	73	27	37

(*D*) *The tuberculous patients at discharge (257 cases)*

	Number of individuals			
	67	55	88	47
mm.	*per cent*	*per cent*	*per cent*	*per cent*
0–2			27	8
2.1–5	14	11	35	34
5.1–8	30	11	17	22
8.1–11	15	18	8	11
11.1–14	6	10	4	8
14.1 and up	35	50	9	17

with a capillary pipette to 100 mm. mark into a shorter tube (2 x 130 mm.) sealed at one end. After one hour at 37.5°C. a reading of the sedimentation rate is made. The tube is then centrifuged for 20 minutes at 3000 RPM and the height of the packed cells is recorded. A corrected sedimentation rate is estimated from the formula:

$$\frac{\text{mm. first hour} \times 100}{\text{plasma volume in mm.}} = \text{per cent}$$

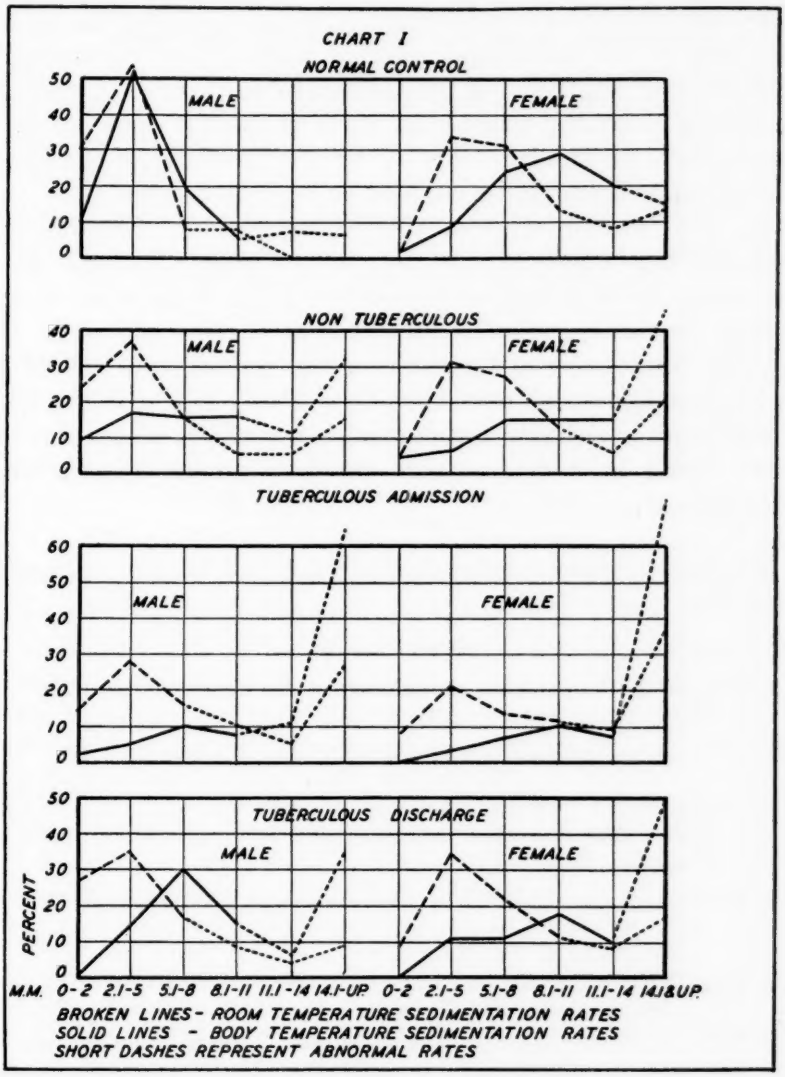
The cell volume is estimated by the formula:

$$\frac{\text{mm. of packed cells} \times 100}{80} = \text{per cent}$$

since 100 mm. of oxalated blood represents only 80 mm. of original blood. Although the first hour reading alone is sufficient in most cases these modifications will give added information to the usual sedimentation test.

Since standards for sedimentation rates of normal persons using the semimicrotechnic and the body temperature method are not now available in the literature, we present in table 7, comparative data on the sedimentation rate at room and body temperatures obtained from a carefully selected group of 380 normal males and females employed in our institution. In addition, the rates for 671 non-tuberculous and 385 tuberculous patients on admission are listed. The discharge sedimentation rates are given for 257 tuberculous individuals. The ages of these groups varied from 20 to 60 years. From the graphs in chart 1, one may establish that when the incubator temperature and semi-micromethod is used the upper limit of normal sedimentation rate in one hour is 11 mm. for males and 14 mm. for females. When using the body temperature technic the percentages of normal rates in the normal control correspond fairly closely with those of room temperature method. In the pathological groups the body temperature method gives a higher percentage of increased sedimentation rates than the room temperature method, indicating a more delicate reaction when the former method is used. In carrying out this study it was noted that the sedimentation rate in apparently

normal healthy females varied more than in males. In some of the females the menstrual period seemed to exert considerable



influence on this phenomenon. If an increased sedimentation rate was present during the menses, its return to normal usually occurred in about a week after cessation of the period.

SUMMARY

In a routine test as simple as the sedimentation test it would seem advisable to adopt a uniform technic so that the results obtained by one investigator may be more accurately compared with those of another. If the sedimentation tube is placed in a vertical position the altitude at which the test is done has little influence on the result. Care in the manipulation of the blood is important; it should be handled as little and as gently as possible. An isotonic solution of anticoagulant is preferable if a large number of tests is to be done at one time. For sedimentation test and cell volume determination 1.1 per cent potassium or sodium oxalate solution is recommended. If comparable and uniform results are to be obtained the temperature should be maintained at a standardized level, preferably 37.5°C. The body temperature is strongly recommended because it places the colloidal blood in a temperature which approaches its natural environment.

We wish to express our appreciation to Mr. E. G. Mullen for his technical assistance.

REFERENCES

(1) CUTLER, J.: The graphic presentation of the blood sedimentation test. Am. Jour. Med. Sc., **171:** 882–901. 1926.

(2) CUTLER, J.: Finger puncture method for blood sedimentation test. Am. Jour. Med. Sc., **173:** 687–694. 1927.

(3) FAHRAEUS, R.: Über die Ursachen der verminderten Suspensionsstabilität der Blutkörperchen während der Schwangerschaft. Biochem. Ztschr., **89:** 355–364. 1918.

(4) GORDON, M. B., AND COHN, D. J.: The effect of external temperature on the sedimentation rate of the red blood corpuscles. Am. Jour. Med. Sc., **176:** 211–214. 1928.

(5) MAGATH, T. B., AND HURN, M.: Concerning anticoagulants. Am. Jour. Clin. Path., **5:** 548–567. 1935.

(6) MAIA, CELESTINO: Causes d'erreur dans la détérmination de la vitesse de sédimentation du sang: verticalité des tubes, température. Compt.-rend. Soc. de biol., **103:** 838–840. 1930.

(7) MORRISS, W. H., AND RUBIN, E. H.: The sedimentation reaction of erythrocytes. Jour. Lab. and Clin. Med., **11:** 1045–1052. 1926.

(8) PLASS, E. D., AND ROURKE, M. D.: A new procedure for determining blood sedimentation rates. Jour. Clin. Invest., **5**: 531–539. 1928.

(9) PINNER, M., KNOWLTON, K., AND KELLY, R. G.: Sedimentation rate of erythrocytes; its relation to fibrin value and cholesterol content and its application in tuberculosis. Arch. Path., **5**: 810–827. 1928.

(10) SCALA, ENRICO: Velocita di sedimentagione degli eritrociti alle varie altezze e a bassa temperatura. Studium, **21**: 15–18. 1931.

(11) VELICOGNA, A.: Sull'influenza della temperatura sulla velocita di sedimentazione dei globuli rossi. Boll. d. Ist. sieroterap. milanese, **9**: 64–70. 1930.

(12) WALTON, A. C. R.: Erythrocyte sedimentation test; clinical and experimental study. Quart. Jour. Med., **2**: 79–115. 1933.

(13) WESTERGREN, A.: On stability reaction of blood in pulmonary tuberculosis. Brit. Jour. Tuberc., **15**: 72. 1921.

(14) WINTROBE, M. M.: Macroscopic examination of the blood. Am. Jour. Med. Sc., **185**: 58–71. 1933.

(15) WINTROBE, M. M., AND LANDSBERG, J. W.: Standardized technique for blood sedimentation test. Am. Jour. Med. Sc., **189**: 102–115. 1935.

USE OF THE STAB CULTURE IN THE GROWTH OF CERTAIN PATHOGENIC FUNGI

JOHN W. WILLIAMS

Homberg Memorial Infirmary and Department of Biology and Public Health, Massachusetts Institute of Technology, Cambridge, Massachusetts*

The object of this work was to determine whether differential characteristics of certain pathogenic fungi and the two Saprophytes, *Lichthemia sp.* and *Scopulariopsis brevicaulis*, not apparent on slants could be augmented or brought out by planting these organisms on and making stabs. It has been noticed[1] that depth of medium is important in bringing out growth of organisms. The depth of the stab medium was approximately 4 cm.

The mediums used were Sabouraud's proof medium and W medium (4 per cent peptone, 1 per cent dextrose, 1.5 per cent agar, pH 5.6). The plants were incubated at room temperature in diffused light over a period of forty days. A brief description of salient features is recorded in table 1.

I have not gone into detail about the stabs since as a rule growth was sparse along these. In the depth of the medium the bits of growth showed little if any growth. Apparently the medium here was not suitable for growth.

A detailed study of growth of the above organisms on slants of W and Sabouraud's proof medium has been published elsewhere[2,3]. In this study I emphasize the use of the stab. It brings out the cup type which some organisms display. The cup in this medium is similar to that often formed as a pellicle on W and Sabouraud's liquid mediums[5]. It emphasizes the organisms' ability to maintain a characteristic dependent on the ratio of dextrose to peptone and possibly dependent at least to a degree on the variation of surface tension in these mediums[4].

*Contribution No. 78.

TABLE 1

SPECIES	CHARACTER AND COLOR OF GROWTH ON	
	William's medium	Sabouraud's medium
Achorion schoenleini	Raised, wrinkled, low white mycelium	Cupped, slightly corrugated at edge, irregular centrally, low white mycelium
Acladium castellani	Fawn, concentric, radiate, raised	Smooth white edge with slightly raised irregular mound of black forming centrally
Candida candida	Poor along stab, surface peppered with small holes	Inverted fir tree subsurface growth, surface irirregularly radiate, folded, slightly raised, waxy centrally
Endodermophyton indicum	Slightly raised, radiate, folded, almost waxy	Suggestion of cupping, irregularly radiate centrally, thin fuzzy mycelium
Endodermophyton tropicale	Delimited, raised cream-puff like	Irregular parchment like growth
Endomyces capsulatus	2 mm. waxy spicule like growth	1 mm. smooth, waxy, bits of low, downy mycelium
Endomyces dermatitidus	Low compact, white mycelium	Low compact, white mycelium
Epidermophyton cruris	Wrinkled, irregular, somewhat cupped centrally, low mycelium	Concave, cupped, low compact mycelium
Epidermophyton inguinale	Somewhat folded, slightly raised, low mycelium	Somewhat cupped, suggestion of corrugation at edge, low mycelial
Epidermophyton rubrum	Folded on self, raised centrally	Mycelial with practically no pigment
Glenospora gammeli	Somewhat waxy, matted, suggestion of pink below and yellow above	Slightly cupped, mycelium smooth and compact, tinted pink below, yellow above
Geotrichum bachmann	Somewhat raised, coarse, sandpapery, slightly irregular	Almost smooth, pink tint
Indiella americana	Doe-skin like, slightly cupped, faintly irregularly radial	Slightly cupped, low mycelial, slightly irregular, olive tinted
Lichthemia sp.	Lighter tint	Darker tint

TABLE 1—*Continued*

SPECIES	CHARACTER AND COLOR OF GROWTH ON	
	William's medium	Sabouraud's medium
Microsporon audouini	Slightly undulating low, tan, fuzzy mycelial, amber color imparted to medium	Suggestion of cupping surface mycelium, tint of amber especially to base
Microsporon felineum	Cupping, dainty growth, corrugated edge, longer mycelium in cup covering base	Cupping somewhat irregular cracked beneath suggestion of corrugation, low white mycelium
Microsporon gypseum	Irregularly radial, cupped, tan tinted powder, yellow amber base	Concave cupping, brown and yellow speckled base
Monilia albicans	Fine lace like	Coarser intertwined growth
Monosporum apiospermum	Concave cupping, low white mycelium	Black base, low white mycelium, suggestion of cupping
Oöspora humi	Similar, little wetter	Similar
Scopulariopsis brevicaulis	Waxy, area of white compact mycelium	Powdery, mycelium light color
Sporotrichum schenkii	Finely interlaced irregular surface	Surface almost smooth waxy
Trichophyton balcaneum	Fine, sandpapery surface	Surface slightly smoother
Trichophyton crateriforme	Irregular, wrinkled, folded, finely cracked, slightly raised	Low, white mycelium, undulating, large cracks centrally
Trichophyton granulosum	Very irregular, wrinkled, partly denuded, cratered, almost like wrinkled scar of burn	Suggestion of cupping, low mycelium, some port wine color at edge and base
Trichophyton decalvans	Similar, slightly more waxy	Similar
Trichophyton gypseum asteroides	Slightly cupped, irregular, undulating, low white mycelium	Concave cupping, somewhat irregular and cracked centrally, base pink color imparted to medium
Trichophyton gypseum lacticolor	Cupping, almost nodular centrally, low white soft mycelium	Concave cupping, some pink at edge and base
Trichophyton interdigitale	Low white mycelium, suggestion of cupping, mycelium compact	Slight concave cupping, tall, white mycelium

TABLE 1—*Concluded*

SPECIES	CHARACTER AND COLOR OF GROWTH ON	
	William's medium	Sabouraud's medium
Trichophyton japonicum	Fine sandpapery, waxy surface	Slightly cupped, faintly amber powder, tint of orange to base and medium
Trichophyton louisiana	Spider web like low mycelial, faintly undulating surface, tint of tan to growth and medium	Low fine mycelium, tint of pink and yellow to base
Trichophyton niveum	Slight cupping, low compact white mycelium	Tall white mycelium, cupping, medium somewhat liquid at base which is dull olive and slightly irregular
Trichophyton purpureum	Slightly irregular, tinted yellow, waxiness at edge, compact white mycelium centrally	Radially concave cupping low faintly brown tinted mycelium, port wine base
Trichophyton sulfureum	Somewhat raised, irregular, undulating, cratered centrally, low powdery mycelium	Beret type growth, cap suggesting radii, being slightly cratered centrally, surface powdery
Willia anomala	Inverted fir tree, more pronounced	

REFERENCES

(1) Hruszek, H.: Über den Einfluss der Nährbodenmenge, des "Lichtes" und der verimpften "Keimzahl" auf das Wachstum von Pilzen. Dermat. Ztschr., **70**: 197–201. 1934.

(2) Williams, J. W.: I. Effect of variation of ratios of dextrose to peptone on colonies of certain pathogenic fungi. Arch. Dermat. and Syph., **32**: 893–914. 1935.

(3) Williams, J. W.: In press.

(4) Williams, J. W.: In press.

(5) Williams, J. W., and Southworth, W.: Unpublished data.

BORDERLINE SILICOSIS*

HENRY C. SWEANY

From the Research Laboratories of the City of Chicago Municipal Tuberculosis Sanitarium, Chicago, Illinois

During recent years industry has been justifiably concerned by claims for damages because of injuries alleged to have come from the inhalation of dust. While all too many of these claims are fraudulent, many occupations do cause a hazard to the health of the worker. Before order can come out of the chaotic state of affairs in this field of endeavor, it will be necessary for the merits of every doubtful case to be established upon the fundamental sciences of pathology, bacteriology, physics, and chemistry, as well as the better known clinical and roentgenological findings. Definite advances in the knowledge of the subject have gradually accumulated, but only in the last twenty years have the various types of pneumoconiosis become systematically studied. Mavrogordato, Simson, Watkins-Pitchford, Gardner, Kettle, Policard and Cummins are a few of the leaders who have made contributions to this knowledge. There are certain types of pneumoconiosis, however, especially the relative components in mixtures of various types of dust with or without tuberculosis that still need investigation.

The term pneumoconiosis, meaning dusty lung, was first used by Zenker[8]. There are valid reasons to continue to use it in a general sense for the whole field of dust diseases and subdivide it into four main sub-groups, depending upon the causative agent and the effect produced.

The most important criterion for division is the presence or absence of a peculiar development of fibrous tissue. The substances producing this "specific fibrosis" are silicon dioxide, sili-

* Read before the Fourteenth Annual Convention of the American Society of Clinical Pathologists held at Atlantic City, June 7 to 9, 1935.

cates, and asbestos. This group may be called *coniofibrosis*, and includes the diseases silicosis, silico-tuberculosis, and asbestosis. A second group of dusts do not produce specific fibrosis, but a mechanical blocking of lymphatics or an irritation that results in a late involvement with bacteria. I wish to suggest for this group the term *coniolymphstasis*. Coal and iron are illustrations, and the pure diseases produced are termed anthracosis, siderosis, et cetera. One must also recognize dusts that are irritative or toxic, the group of which may be termed *coniotoxicosis*. They damage directly or are proteins that sensitize the tissues to subsequent exposures. Corrosive dusts may also be included in this heading. Finally, in pneumoconiosis, most commonly encountered, there are two or more agents mixed in varying proportions with different kinds and degrees of infection. These mixed groups vary within wide limits, depending upon the amount and character of the dust which in turn regulates to a certain extent the bacterial flora. For example, coniofibrosis is prone to develop tuberculosis, while coniolymphstasis tends more to acute infections.

Of these many varieties, most of them are sufficiently characteristic to permit of a diagnosis by men familiar with the subject long before any extensive disability occurs. When tuberculosis is a complication, however, an early diagnosis is difficult if at all possible. This disease not only gives a sort of coup de grâce to advanced silicosis, but appears to be more fulminating in early silicosis. This seems to be bourne out also by the work in animals by Gardner[3] and Kettle[4]. The only difficult problem, therefore, is in borderline cases that result in a disabling silico-tuberculosis. As the tuberculosis advances, the physical and X-ray findings become more and more like tuberculosis, until it is difficult or impossible to distinguish them from tuberculosis until an autopsy and chemical examination is performed.

¶The meaning of borderline silicosis, as used herein is to be understood as the least amount of pathological change that is pathognomonic of the condition. The various means of determining the presence or absence of a silicosis in the various stages, will be taken up in proper sequence.

Of the factors useful in diagnosis, the first and perhaps most important is the history. Certainly, with no history of exposure, the diagnosis cannot well be pneumoconiosis, irrespective of appearance, but nevertheless the amount of exposure is not necessarily related to the stage of the disease.

Physical and roentgenological findings in certain advanced cases are presumptive evidence, and up to the present afford the chief antemortem criteria. In borderline types, however, neither is of much value.

The chemical findings during life are useful if found abnormal, as demonstrated by Böhme and Kraut[1] and King and Dolan.[5] If there is sputum, a determination of silica will give a fair idea of the amount of silica in the lung, and the micro-incineration and petrographic analysis will sometimes aid in the determination of its quality. In early cases, however, there is not enough increase in silica to be found chemically, and the only way any solution of such a problem can be made is after death and a careful necropsy.

Pathologically, the silicotic nodule is quite characteristic, but when there is an overlapping with tuberculosis, anthracosis, or some other related condition, the whole picture is clouded. The fewer the nodules the more the confusion until it becomes impossible to tell whether any silicosis exists at all by a gross pathological examination. In the microscopic study, if there are typical silicotic whorls throughout several of the lymph nodes, it is not difficult, but if the nodules do not have typical whorls and the fibrils are slurred or caseous, it sometimes becomes difficult to differentiate from uncomplicated tuberculosis. Definite specific nodules are enough to establish a diagnosis of themselves, but where they are distorted with tuberculosis, the history of exposure is also necessary as well as a confirmation by chemical examination.

If there is less than 2.0 mgm. of silica per gram of dried lung parenchyma, the silicotic nature of any nodule is dubious. With more than that quantity in the lung parenchyma, specific nodules usually make their appearance in the lymph nodes to be followed later in the parenchyma. In coal miners and certain other mixed dusts the threshold of nodulation is sometimes higher. The chemical analysis of the lymph nodes alone is of little value, be-

cause in normal adults there is usually an accumulation of silica up to 6.0 and 7.0 mgm. per gram of dry tissue as the patients reach adult life.

It may be observed in analyzing the preceding facts, that there are two types of criteria presented, namely, the presumptive, including an indefinite exposure, the physical examination, and the X-ray; and the critical or absolute, including the history of definite exposure, the pathology and the chemical findings.

This report aims to deal with the absolute data and assumes that the differential diagnosis has been established. It is, in brief, an attempt to reveal the threshold of silicosis and its relationship with tuberculosis. Five cases are submitted as illustrations.

CASE REPORTS

Case 1 was a 27 year old lead and zinc miner who worked for eight years during the time when safety devices were used. In the lung specimen there were no gross nor microscopic silicotic nodules, but a typical fibrocaseous and ulcerative sub-apical tuberculosis. There was only 1.9 mgm. of silica per gram of dried lung. The patient's father, mother, and two sisters died of pulmonary tuberculosis. This was ordinary tuberculosis without any question of silicosis or silico-tuberculosis as a contributing cause of death.

Case 2 was a 56 year old American who had worked for the last seventeen years as a barber. He had worked as a hoisterman and shaft-sinker for twelve years before he took up barbering. There was a history of syphilis of sixteen years duration, for which there had been little treatment. Death was due to tuberculosis. The pathology was strictly that of a sub-apical tuberculosis, although there was a moderate amount of pigment in the lymph nodes and a few phagocytes that were arranged in plaques; there were no fibrotic whorls nor even linear fibrosis.

Case 3, a 42 year old Armenian, spent six years as a moulder in a brass foundry, but quit work in April, 1932 due to a disability lawsuit which was settled out of court for a reputed sum of $3,000. The onset of his terminal disease was hemorrhage in September, 1932. A physician took an X-ray and pronounced him tuberculous. The findings were characteristic of tuberculosis in every way. There was a mild polymorphonuclear leukocytosis ranging from 8,000 to 11,000, and his sputum was positive for tubercle bacilli.

In the lung were huge cavities in both the right and left upper lobes and some smaller ones in the lower lobes. There was acinonodose tuberculosis in the upper halves of both lower lobes and an occasional small black area throughout the mid section of the lung. The bronchial and hilum lymph nodes were black but offered no resistence to the knife. There was no gross evidence of silicosis.

The microscopic appearance of a lymph node, taken from the hilum of the

lung, showed a few nodules resembling tuberculo-silicotic whorls, and a large number of caseous tubercles. In the bronchopulmonary nodes out from the hilum there was revealed a marked amount of pigmentary accumulations but no fibrosis. Under the lymph node capsule, however, there was a moderate accumulation of pigment and a beginning linear fibrosis, but no whorls. Other slides, taken at random throughout the parenchyma, revealed a preponderance of tuberculosis. There were a few questionably silicotic tubercles that had become markedly caseous. There was also a marked emphysema in the outlying lung tissue with numerous recent typical caseous-nodular tubercles.

This specimen seemed to reveal the earliest recognizable type of silicotic nodulation in lymph nodes, with a few questionable tuberculo-silicotic nodules in the parenchyma. On careful study the nodules were found to be more tuberculous than silicotic. Furthermore, they were not in relation to the main lymphatic trunks (plate 1). It is thought probable that this patient's silica (4.1 mgm. per gram of dried lung) was not of the type to form specific fibrosis. Even if it were, there was no such fibrosis present; and what is more important, the tuberculosis was overwhelming. It is quite apparent that the tuberculosis was so acute and extensive and the silicosis too slight to consider at the time of death a diagnosis of silico-tuberculosis.

Case 4, was a 43 year old lead and zinc miner from southeastern Missouri. There was no accurate history obtainable because the patient died in a gastric hemorrhage. Apparently there had been no pulmonary complaint. From the lung specimen it was evident that there was some contact with dust.

The ante mortem roentgenogram presented a peribronchial fibrosis or "arborization," with very fine mottling, more like an early anthracosis (plate 2, fig. 1). The arborization extended out to the pleura. There was a slight exaggeration of the hilum lymph nodes in size and density. There was a constriction of the chest about the mid zone that suggested a fixation of the ribs from this region towards the base.

The gross pathology revealed a black lung with considerable fibrosis and emphysema. There were small foci of black pigment scattered throughout. The hilum nodes were large and black, many measuring 2 x 3 cm.

Microscopically, a hilum lymph node revealed almost solid masses of phagocyted black pigment throughout, with a few regenerated follicles of lymphoid tissues. In the parenchyma there was revealed a lymphatic congestion of all lymph vessels with phagocyted black pigment (plate 2, fig. 2). In the larger foci there was a moderate amount of fibrous tissue but none of it in the whorls. There was also a marked amount of emphysema.

The history of mining in the lead and zinc mines of Missouri was evidence of exposure to rather pure silica, and the silica content of 3.4 with a low hydrofluoric acid residue, revealed the fact that he had such exposure. The exposure, however, was either not long, was recent, or he had good elimination to prevent the development of fibrosis. As he was only 43 years of age, it may well be taken as a pre-fibrotic stage of silicosis, modified by the carbon pigment found

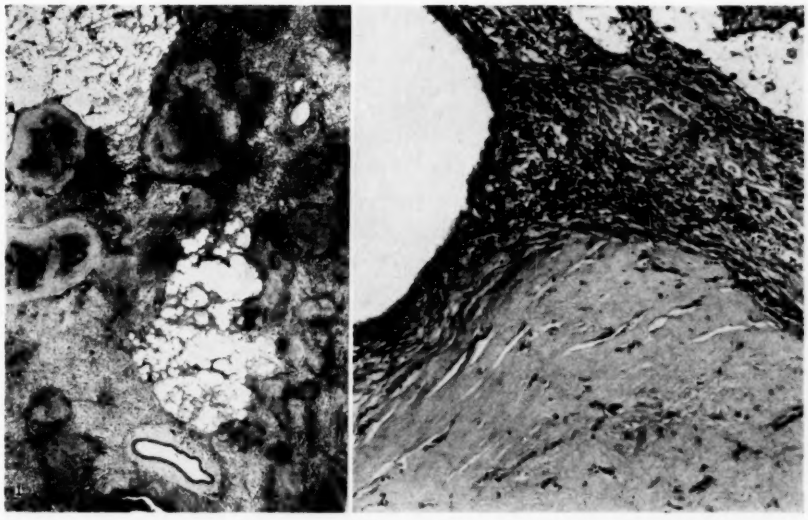


PLATE 1

FIG. 1. LOW POWER PHOTOMICROGRAPH OF THE LUNG OF CASE 3. Nodular tubercles which resembled silicotic tubercles proved to be more tuberculous than silicotic. × 7.

FIG. 2. A HIGHER POWER OF ONE OF THE TUBERCLES.

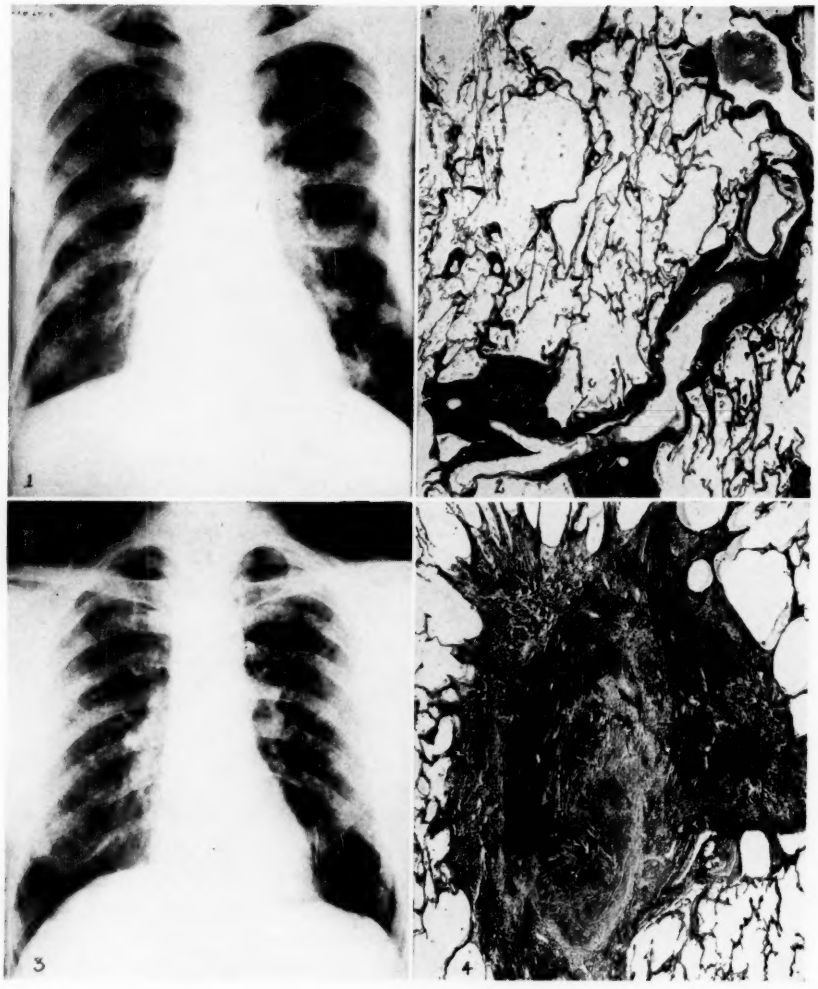


PLATE 2

FIG. 1. ROENTGENOGRAM OF CASE 4. A "pre-silicotic" lung in a lead and zinc miner, revealing a few very fine densities and increased arborization.

FIG. 2. A LOW POWER PHOTOMICROGRAPH OF THE LUNG. Note the tremendous engorgement of the lymphatics with phagocyted pigment. × 23.

FIG. 3. ROENTGENOGRAM OF CASE 5. A first stage silicosis, farther advanced than the preceding.

FIG. 4. A PHOTOMICROGRAPH OF ONE OF THE SMALL LUNG NODULES SHOWING THE BEGINNING FIBROSIS WITHIN THE PIGMENTED FOCI. × 200.

in the quartz in the mines in which he worked. There is little doubt that carbon delays and masks the development of silicosis. This was observed in the lead and zinc miners in earlier studies[7] and has been reported formerly by Cummins[2] and Sampson[6] in coal miners. Had the patient lived another ten years, typical fibrosis would perhaps have made its appearance.

This case illustrates the fact that much pathology may exist that cannot be determined or even anticipated on the X-ray. The history of inhaling a dangerous dust is more important in such cases. It shows also that disease may not develop for a decade or more after exposure, and that a complete solution of the problem will be to keep the dangerous dusts out of the lungs. Then the extra hazard of contracting tuberculosis will not exist.

Case 5, was a 55 year old patient of Dr. William M. Kinney, and information is furnished through the courtesy of Dr. Jesse Douglass of Webb City, Missouri. His occupation was a tourist resort operator and owner, but he had been a lead and zinc miner in Webb and Joplin, Missouri for many years. He had not worked in the mines, however, for fifteen years. In March, 1934 he consulted Dr. Kinney, complaining that for the past three weeks he had been having a "cold in the head" which was followed by a cough and slight expectoration. The physical examination of the lungs showed no abnormal findings. The X-ray film showed a moderate amount of mottling, evenly distributed throughout both lungs, and appeared to radiate outwards from the hilum. The diagnosis at that time was "acute coryza followed by mild bronchitis, with a moderate amount of silicosis throughout both lungs." Within two weeks he recovered and felt well. There were no symptoms of silicotic disease, no cough, dyspnea, nor "black spit." In October, 1934 he was found dead in a tourist cabin from carbon monoxide poisoning.

The roentgenological findings show fine nodules throughout the parenchyma, not only out from the hilum but all the way to the ribs, and from the apex to the base (plate 2, fig. 3). The nodules vary in size and density, ranging from the tiny flake-like shadows seen in anthracosis to a few nodules 3 to 4 mm. in diameter. The larger nodules are too widely scattered to permit a diagnosis of silicosis on their own account. The hilum nodes are enlarged and undergoing calcification, which fact suggests a silico-tuberculosis. Such appearances should arouse suspicion of this disease in any male past middle life. The post mortem roentgenogram reveals a bullous emphysema over the uppers; a fine nodulation with diffuse cloudy infiltration; and massive lymph node calcification at the hilum, involving most of the lymphatic nodes of the lungs and mediastinum.

A necropsy was performed by the coroner, Dr. W. G. Hogan, and Dr. William M. Kinney. The musculature was a cherry red color. All the organs were in a poor state of preservation. The lungs were studied in our laboratory and found to be soft and black with marked emphysema. Deep in the lung tissue could be felt numerous small nodules about 2 mm. in diameter. On section there are numerous black concrete nodules throughout the parenchyma. Some contain early fibrosis (plate 2, fig. 4). These nodules, of about 3 mm. in

diameter, were scattered out from the hilum and were placed on an average of 2 cm. apart in the parenchyma of the lungs. The bronchial and tracheobronchial lymph nodes were enlarged, black, and contained much calcification and specific fibrosis. The chemical content of 3.2 mgm. per gram of dried lung tissue was definitely pathological.

This is another accidental death in a slightly more advanced and more definite stage of silicosis, than the preceding. There are definite fibrous whorls in the small nodules, and there are calcifications in the hilum nodes, characteristic of silico-tuberculosis. The history is the most important factor in the ante mortem findings of this case. The physical findings were essentially negative. The roentgenogram is that of a first stage silicosis due to the enlarged and calcified hilum nodes. The mottling is more characteristic of anthracosis or of a non-fibrous pneumoconiosis. The pathological and chemical findings remove all doubt as to the nature of the process.

DISCUSSION

It is clear from these cases that in minimal silicosis alone there is no disability and no life hazard. When tuberculosis intervenes, however, the problem becomes a serious one and the question is whether silica or silicosis acts as a "wet nurse" to the tuberculosis. The location, extent and acuteness of the tuberculosis will help to determine this. In the first two cases there was not a pathological content of silica present, and there was not enough pathological evidence of silicosis to ascribe the death in any way to it. In the third case the silica was pathological in content, but there were no specific lesions present. Furthermore, the tuberculosis was sufficiently extensive and acute to account for the damage in its own right. In the fourth case there was also a pathological level of silica, but no signs of fibrous whorls pathologically. There was a tremendous engorgement of all lymphatics, however, that has been found to result in silicosis in other miners from this region. It was thought probably to be a pre-fibrotic stage of silicosis, entailing no hazard of itself, but always a potential danger of becoming tuberculous. With a history of exposure to a dangerous dust, such findings are significant. Such types are only found, however, in accidental deaths, and offer no problem themselves. The fifth case was clearly silico-tuberculosis.

CONCLUSIONS

From this work one may conclude that in the pneumoconioses only a complicating tuberculosis offers any problem that cannot be dealt with during life by adequately trained medical men. In the event of a complicating tuberculosis, no decision is possible without a necropsy and a post mortem pathological and chemical examination. As a minimum requirement for a diagnosis in these cases there should be a definite history of exposure to a sufficient quantity of a dust that is known to produce disease; pathological evidence of silicotic fibrosis in several regions of the lymphatics or parenchyma, or both; and a chemical content of 2 mgm. of silica per gram of dried lung parenchyma. It must be further stressed that these criteria must be established by adequate scientific examinations.

REFERENCES

(1) Böhme, A., and Kraut, H.: Über den Kieselsäuregehalt des Blutes von Staublungenkranken. Arbeitsphysiol., **5:** 269–277. 1931.

(2) Cummins, S. L.: Effects of coal dust upon the silicotic lung. Jour. Path. and Bacteriol., **30:** 615–619. 1927.

(3) Gardner, L. U.: Studies on experimental pneumonokoniosis. V. The reactivation of healing primary tubercles in the lung by the inhalation of quartz, granite, and carborundum dusts. Am. Rev. Tuberc., **20:** 833–875. 1929.

(4) Kettle, E. H.: The interstitial reactions caused by various dusts and their influence on tuberculous infections. Jour. Path. and Bacteriol., **35:** 395–405. 1932.

(5) King, E. J., and Dolan, M.: Silicosis and metabolism of silica. Can. Med. Assn. Jour., **31:** 21–26. 1934.

(6) Sampson, H. L.: Silicosis symposium. New York: Saranac Lake, 1934.

(7) Sweany, H. C., Douglass, Jesse E., and Porsche, Julius: In press.

(8) Zenker, F. A.: Ueber Staubinhalationskrankheiten der Lungen. Deutsch. Arch. f. klin. Med., **2:** 116–172. 1866–67.

STUDIES OF UROBILINOGEN. I*

An Improved Method for the Quantitative Estimation of Urobilinogen in Urine and Feces

CECIL JAMES WATSON

Department of Medicine, University of Minnesota Hospital, Minneapolis

Since the early investigations of Friedrich v. Muller[17], urobilinuria has been widely believed to be a sign of disturbed liver function. The detailed experimental studies of McMaster and Elman[3,4,14,15,16] provided evidence to substantiate this belief. The outstanding clinical and experimental study of Wilbur and Addis[35] in 1914 emphasized the importance of urobilinuria as an evidence of liver dysfunction. This work also demonstrated the usefulness of urobilinogen estimations in the feces, particularly in the anemias. These findings were corroborated by Eppinger[5], Fischler[10,11], Adler and Bressel[1], Wallace and Diamond[23], Lichtenstein and Terwen[13], Weiss[34], Royer[18,19], and the writer[24]. Saillet[20], and Wilbur and Addis noted a considerable fluctuation in urinary excretion of urobilinogen from day to day, and at various times of the day, in patients having liver disease. Bang[2] observed hourly variations in amount related to ingestion of food in normal individuals. All of these variations have undoubtedly lessened the value of qualitative tests such as the Schlesinger and Ehrlich reactions, and also of roughly quantitative procedures such as those of Wilbur and Addis, and of Wallace and Diamond, because of the varying dilution of the urine. It is clear that knowledge of the per diem excretion is necessary if the maximum value of urobilinuria as a liver function test is to be realized. It is likewise certain that tests carried out on individual stool specimens, whether qualitative or quantitative, have little value, except for determining the presence or total absence of urobilin

* Aided by a grant from the research fund of the Graduate School University of Minnesota.

or urobilinogen. The work of Singer[21] in particular demonstrated the daily fluctuations in urobilinogen excretion in the feces particularly when the patient under investigation was not receiving a constant diet. Apart from this source of variation, the variability in frequency of bowel movements in different individuals makes imperative the determination of the average amount excreted over several days. Numerous methods for the quantitative estimation of urobilin, urobilinogen, or both have been proposed. These were discussed in a previous publication,[24] and at the same time a modification of Terwen's[22] quantitative procedure was described. The principle of Terwen's method is the back reduction of preformed urobilin to urobilinogen by means of ferrous hydroxide followed by a colorimetric determination in which the Ehrlich reaction is employed. The color comparison is made with a standard alkaline phenolphthalein solution. Terwen had only an amorphous urobilin with which to standardize the color relationship. For this purpose the writer used crystalline mesobilirubinogen obtained by the reduction of bilirubin according to H. Fischer's[7] method. Mesobilirubinogen was shown by H. Fischer and Meyer-Betz[8] to be identical with crystalline urobilinogen as isolated from human urine. Prior to 1932 little was known of corresponding substances in the feces, except of course, that a relatively large amount of Ehrlich reacting substance was regularly present. During unsuccessful attempts to isolate urobilinogen from human feces in 1932, it was found that crystalline stercobilin could readily be isolated.[25,26,30] In 1933 crystalline urobilin was isolated from pathologic human urines and this has been shown to be identical with stercobilin.[27,28,31,32]. Recently[33] it was shown that these crystalline substances are not products of mesobilirubinogen, but of another chromogen also having urobilinogen characteristics. Fortunately for the quantitative estimation of urobilinogen this second chromogen has been found to given an Ehrlich reaction of approximately the same intensity as that of mesobilirubinogen. Evidence for this is presented later in this communication. When crystalline urobilin was isolated it was shown[28] that it is not the only derivative of urobilinogen, but is regularly accompanied in both feces and urine

by a pigment which was named copromesobiliviolin because of its extreme similarity to mesobiliviolin, a derivative of mesobilirubinogen obtained in vitro by H. Fischer and Niemann[9]. Copromesobiliviolin is characterized by a violet color, spectroscopic absorption in the region of 600 $\mu\mu$, and red fluorescence with alcoholic zinc acetate. This substance is always present in considerable amount in extracts of feces, and as yet little is known as to whether its formation is at the expense of that of urobilin, and as to the relative amounts of the two substances in different feces and in different urobilin containing urines. Certain information suggesting a close relationship between the two substances has been obtained and will be presented in a separate communication.

From this it is evident that methods of estimation based upon either the absorption spectrum of urobilin, or its green fluorescence with zinc salts cannot be relied upon to give accurate information as to the amount of urobilinogen originally present. For this reason the estimation of urobilinogen itself is preferable.

The purpose of the present investigation was to improve and simplify the previously described method with the aid of newer information concerning both urobilin and urobilinogen. The improved method described below has been employed in the study of urobilinogen excretion in urine and feces in 205 individuals, of whom 135 had jaundice or disease of the liver. In a considerable number of this group, urobilinogen excretion was compared with galactose tolerance and bromsulphalein retention, as an index of liver function. The results of these studies are the subject of separate communications to be published elsewhere. Briefly they show that knowledge of the per diem excretion of urobilinogen is essential to the clinical study of jaundice and liver disease, and it appears that the quantitative estimation of urobilinogen in the 24 hour urine is more reliable in determining liver function than is the galactose or bromsulphalein test.

DESCRIPTION OF METHOD

The improved quantitative procedure now in use is as follows:

1. Feces. (a) Preparation of sample. The entire amount for a four day period is collected in an opaque container which is kept in a cool place during this period of collection. A quart waxed paper carton serves very well for this

purpose. Details regarding the collection of feces have already been given.[24] The four day amount is weighed and thoroughly mixed. If the consistency of the feces permits, this mixture is accomplished in the waxed paper carton directly, otherwise it must be carried out in a large mortar. If the material is partly liquid, partly solid, it has been found advantageous to pour off the liquid and carry out separate determinations on liquid and solid portions. Ten grams of the mixed feces are weighed out on a small filter paper, or if the feces are not sufficiently dry, in a small evaporating dish. This amount is thoroughly ground into a paste with 10 to 20 cc. of a 90 cc. amount of water in a mortar. The remainder of the water is then added, and after further grinding, the mixture is allowed to stand briefly. The supernatant fine suspension of feces is poured into a 1000 cc. Erlenmeyer flask containing 100 cc. of 20 per cent ferrous sulphate ($FeSO_4 \cdot 7H_2O$) solution (20 grams of the ground crystals and 92 cc. distilled H_2O). The remaining material in the mortar is again ground with small amounts of distilled H_2O, all of which are poured into the large Erlenmeyer Flask until a total of 200 cc. more have been used, the mortar finally being washed clean. One hundred cubic centimeters of 10 per cent NaOH are now added with shaking. The mixture is corked and set aside in the dark to stand for one hour.

If the stool appears acholic the above procedure is modified so that only 100 cc. of distilled water are used in grinding up the sample, and only 25 cc. each of the 20 per cent ferrous sulphate and 10 per cent NaOH solutions.

(b) Development of urobilinogen-aldehyde color. Two to 3 cc. of the filtrate in a test tube are acidified with the same amount of a modified Ehrlich's reagent: 0.7 gram pure p-dimethylaminobenzaldehyde (Pfanstiehl), 150 cc. concentrated HCl, 100 cc. distilled water. Three to 4 cc. of a saturated aqueous solution of sodium acetate are added and the intensity of the developing color is noted. If the color is very intense 1 cc. or even less of filtrate will suffice in the quantitative determination, if only moderately intense 2 cc.; if pale red, 3 to 5 cc.; if faint, 10 to 30 cc.; if absent, 50 cc. The amount decided upon is placed in a small separatory funnel, after dilution to 20 cc. with distilled water (if less than 20 cc. of filtrate were used), then covered with 20 to 30 cc. of pure petroleum ether and strongly acidified with glacial acetic acid. (The petroleum ether used for this extraction is purified by standing for several days over concentrated H_2SO_4, and further by repeated washing with distilled water followed by distillation.) After acidification the mixture is shaken vigorously for several seconds. Upon separation the aqueous portion is extracted twice more with petroleum ether. The combined petroleum ether solution is washed twice with distilled water. The urobilinogen is then extracted by vigorous shaking with 1 to 2 cc. of the Ehrlich reagent. At least twice as much of a saturated aqueous solution of sodium acetate is now added with resultant development of the color reaction. This is again shaken vigorously. As Terwen has shown, color due to indol or skatol disappears as the hydrochloric acid is changed to acetic acid. Furthermore, any excess of aldehyde returns to the petroleum ether. The petroleum

ether is again shaken with a small amount of Ehrlich's reagent followed by sodium acetate solution. In case more than a very faint color develops this procedure must be repeated until it is certain that the extraction of urobilinogen has been complete.

(c) Colorimetric determination. This should be carried out without delay. The colored solution is made up to a volume convenient in calculation. A portion is placed in the right hand test tube of a Hellige type P.S.P. colorimeter.* If the color is found to be more than 50 per cent of the standard, the solution should be further diluted, as comparisons can be made most accurately between 20 and 50 per cent.

Calculation:

$$\frac{\text{cc. ferrous hydroxide mixture}}{\text{gms. of feces}} \times \frac{\text{cc. final solution}}{\text{cc. filtrate used}} \times \text{per cent of color standard} \times \frac{\text{wt. of feces}}{100} \times \frac{1}{\text{no. of days of collection}} = \text{mg. per day}$$

Examples:

$$\text{Normal feces: } \frac{500}{10} \times \frac{45}{3} \times 0.35 \times 2.8 \times \tfrac{1}{4} = 183.7 \text{ mg.}$$

$$\text{Acholic feces: } \frac{150}{10} \times \frac{10}{40} \times 0.5 \times 4.2 \times \tfrac{1}{4} = 1.9 \text{ mg.}$$

2. Urine. A 24-hour urine specimen is collected in a brown glass bottle containing approximately 5 grams of sodium carbonate and 100 cc. of petroleum ether. At the end of the period of collection the contents of the bottle are poured into a 1000 cc. graduated cylinder, and after standing a few moments, the volume of the 24-hour urine is measured. The entire amount of urine is now separated from the petroleum ether by means of a separatory funnel of 1- or 2-liter capacity. Fifty cubic centimeters of the mixed urine is placed in an Erlenmeyer flask of 300 to 500 cc. capacity, and to this amount is added 25 cc. of 20 per cent ferrous sulphate (5 grams of crystals ground to a powder and dissolved in 23 cc. of distilled H_2O), and, with thorough mixing, 25 cc. of 10 per cent NaOH. After one hour a small portion of the filtrate is tested qualitatively with the Ehrlich reagent as described above. In this way, as for the feces, a desirable amount of filtrate is chosen for the quantitative procedure. If the reaction is entirely negative, 50 cc. of filtrate are used.

The filtrate is extracted three times with petroleum ether in a separatory funnel after acidification with glacial acetic acid. Emulsions may occur but

* Permanent glass standard colorimeter for determinations of phenolsulphonphthalein in urine, obtained from Hynson, Westcott and Dunning Co., Baltimore, Maryland.

are usually readily separated by the addition of a few drops of 95 per cent alcohol. The combined petroleum ether is then washed with water and the urobilinogen extracted in the same way as described above.

Calculation:

$$\frac{100}{50} \times \frac{\text{cc. final sol.}}{\text{cc. filtrate used}} \times \text{per cent of standard} \times \frac{\text{vol. of 24 hour urine}}{100} = \text{mg. in 24 hours}$$

Examples:

$$\text{Normal: } 2 \times \frac{40}{50} \times 0.15 \times 8.5 = 2.04 \text{ mg. in 24 hours}$$

$$\text{Liver disease: } 2 \times \frac{30}{2} \times 0.2 \times 10.5 = 63.0 \text{ mg. in 24 hours}$$

Experiments relative to changes which have been made in the method previously described[24].

I. Standardization of color relationship for colorimetric determination

(a) With mesobilirubinogen. Previously[24], the color of a solution of 0.37 mg. of crystalline mesobilirubinogen with Ehrlich's reagent, diluted with water to 100 cc., was found to be of the same intensity as Terwen's standard alkaline phenolphthalein solution. This color relationship has been studied again in the present investigation with essentially the same result. The urobilinogen-aldehyde color in known concentration was also compared in the P.S.P. colorimeter used in the routine procedure.

Crystalline mesobilirubinogen (urobilinogen) was prepared from pure bilirubin according to the method of H. Fischer[7]. It was recrystallized out of acetic ester and dried in vacuo over P_2O_5. For the following determinations the amounts used were weighed out on a micro-balance sensitive to 0.002 mg. In each instance the substance was dissolved in 1 to 2 cc. of Ehrlich's reagent and transferred to a small separatory funnel. Following the addition of several cubic centimeters of distilled water, 8 to 10 cc. of saturated aqueous solution of sodium acetate were added, and the mixture vigorously shaken with 25 to 30 cc. of purified petroleum ether. The colored aqueous solution was separated into a graduated cylinder. The petroleum ether solution was now repeatedly extracted with small amounts of Ehrlich's solution, each extraction followed by addition of sodium acetate, vigorous shaking, and removal of the colored aqueous solution to the graduated cylinder. After all of the color was extracted, the urobilinogen aldehyde solution, following suitable dilution, was compared in a Duboscq colorimeter with Terwen's standard solution and was also compared in the P.S.P. colorimeter. Following is an example of such a comparison:

1.244 mg. mesobilirubinogen after treatment with Ehrlich's reagent as above

described were diluted further to 200 cc. with distilled water. This solution was compared with Terwen's standard phenolphthalein solution in a Duboscq colorimeter. With the standard at 20 the readings were: 12.5, 11.8, 12.3, 12.1,—average 12.2.

$$\frac{12.2}{20} \times 0.622 = 0.375 \text{ mg. per 100 cc.}$$

For the purpose of comparison in the P.S.P. colorimeter the solution was further diluted with equal parts of water, so that 100 cc. contained 0.311 mg. urobilinogen. This was found to be 42 per cent on the P.S.P. scale.

$\frac{100}{42} \times 0.311 = 0.740$ mg. per 100 cc., the amount equivalent to 100 per cent on the P.S.P. scale

TABLE 1

MILLIGRAMS OF UROBILINOGEN PER 100 CC.

SPECIMEN	TERWEN'S SOLUTION	P.S.P. COLORIMETER
1	0.42	0.652
2	0.443	0.713
3	0.448	0.83
4	0.365	0.77
5	0.364	0.77
6	0.32	0.64
7	0.375	0.740
8	0.365	0.675
Average.....	0.387	0.721

Eight determinations were made in this way with separate weighings, the amounts of urobilinogen varying between 1 and 2 mg. The results are shown in table 1.

These values represent the number of milligrams of urobilinogen per 100 cc., which, with the aldehyde, produce a color of intensity equal to Terwen's standard solution or to 100 per cent in the P.S.P. colorimeter.

Immediately after the above color comparisons were made, the solution in each instance was examined in a Zeiss spectrometer. In none was any appreciable urobilin absorption at 495mμ observed, indicating that transition from urobilinogen to urobilin was as yet negligible.

The value corresponding to Terwen's standard solution agrees with that previously reported[24].

(b) Crystalline stercobilin (urobilin). This was isolated according to the procedure recently described.[30] The hydrochloride was recrystallized four times

from chloroform and dried in vacuo over P_2O_5. Five milligrams were dissolved in 4 cc. of 95 per cent alcohol. This was diluted with distilled water to a volume of 60 cc. and the entire amount was placed in a flask containing 20 cc. of 16 per cent ferrous ammonium sulphate. To this was added slowly and with constant mixing 20 cc. of 12 per cent NaOH. The flask was corked and set away in the dark. At the end of two hours the filtrate was colorless and did not show any urobilin absorption at 510mμ. Four cubic centimeters of the filtrate were subjected to the quantitative procedure described above. The final colored solution, 40 cc. in volume, was compared with Terwen's standard solution in a Duboscq colorimeter. With the standard at 20, the average reading was 14.7. Since this solution contained the equivalent of 0.5 mg. stercobilin per 100 cc., $\frac{14.7}{20} \times 0.5 = 0.367$ mg. per 100 cc. Another determination with 5 cc. of filtrate gave the value 0.38 mg. per 100 cc. These values represent the amount of crystalline stercobilin which, after reduction with ferrous hydroxide, yielded a color with Ehrlich's reagent, and in 100 cc. volume, of intensity equal to Terwen's standard phenolphthalein solution.

From this it is evident that the leuko-compound of stercobilin or urobilin yields a color with Ehrlich's reagent of equal intensity with that of the mesobilirubinogen aldehyde compound. Heilmeyer and Krebs[12] have recently shown that the intensity of absorption of the reduction product of crystalline stercobilin with Ehrlich's reagent, as measured in the spectrophotometer, is the same as that of the mesobilirubinogen-aldehyde compound. They believed that the crystalline substance which they isolated by a somewhat different method differed from that originally described by the writer[25,26,30]. However, the writer has demonstrated[32] that the stercobilins isolated by the two methods are in no way different; the same characteristic crystals of the hydrobromide and of the iron chloride molecular compound were obtained from each, and the crystals of the hydrochloride as well as the free substance were also identical.

In the above determination 16 per cent ferrous ammonium sulphate and 12 per cent sodium hydroxide were used so that the values obtained would be entirely comparable with that given by Terwen, 0.4 mg. per 100 cc. A similar determination was made in which 20 per cent ferrous sulphate and 10 per cent NaOH were used, as in the routine quantitative procedure. Approximately the same value was obtained, 0.39 mg. per 100 cc.

Again there was no spectroscopic evidence of any development of urobilin during the period of the color comparisons.

(c) With copromesobiliviolin. Realizing that this substance regularly occurs in considerable amount in association with urobilin and that it is also a derivative of urobilinogen, it was necessary to determine whether it could be reduced with ferrous hydroxide, and whether the resulting reduction product gives as intense an Ehrlich reaction as is given by urobilinogen and reduced urobilin. The substance was isolated from feces according to the method previously described.[28,29]

It is obtained in the form of what appear to be very minute prisms, but recrystallization has not been possible as the substance is labile, changing into a dark brown pigment having many of the characteristics of copronigrin[26]. The material used for the present determination had an intense violet color with absorption in the region of 600mμ only, and exhibited red fluorescence with alcoholic zinc acetate solution.

1.2 mg. were dissolved in 4 cc. of 95 per cent alcohol and this was further diluted with water to 30 cc. To this was added 10 cc. of 20 per cent ferrous sulphate, and, with constant mixing, 10 cc. of 10 per cent NaOH. The flask was corked and set away in the dark. After two hours the filtrate was clear and quite colorless. The qualitative aldehyde reaction was relatively weak. Twenty-five cubic centimeters of the filtrate was subjected to the same procedure described above, acidification, petroleum ether extraction, extraction with Ehrlich's reagent and addition of sodium acetate. The final colored solution measured 40 cc. With the standard at 10, the average reading was 17.4. Since the solution contained an equivalent of 1.5 mg. per 100 cc., the calculation was $\frac{17.4}{10} \times 1.5 = 2.61$ mg. per 100 cc. This was the concentration producing a color equal in intensity to that of Terwen's solution, and it is thus evident that the leuko-compound of copromesobiliviolin is not capable of producing as intense an Ehrlich reaction as is urobilinogen.

II. Recovery of urobilinogen and of urobilin with the routine quantitative procedure after addition of varying amounts of the crystalline substances to urine and feces

(a) Crystalline mesobilirubinogen (urobilinogen) obtained from bilirubin by H. Fischer's[7] method, and crystalline urobilinogen isolated from human urine by a modification of the original method of H. Fischer and Meyer-Betz. This procedure has been described in a separate publication.[33] For present purposes it is sufficient to observe that the urobilinogen obtained in this way was in all respects identical with mesobilirubinogen obtained in vitro, in confirmation of the findings of Fischer and Meyer-Betz. Prior to use both substances were recrystallized from acetic ester, and were dried in vacuo over P_2O_5, at room temperature.

Varying small amounts were added to urines not containing demonstrable urobilin or urobilinogen, and the routine quantitative procedure was then applied. The results are best shown by means of table 2. As an example of how these values were obtained the calculation for the first of the group is given:

$$\frac{150}{100} \times \frac{18}{2} \times 0.38 \times 0.72 = 3.69 \text{ mg.}$$

A similar determination was made in the following way for the feces. Two portions of the same thoroughly mixed sample of feces were used. This was obtained from a patient with high grade partial common duct obstruction due to

TABLE 2

RECOVERY OF MESOBILIRUBINOGEN

	I	II	III	IV*	V*
Amount of mesobilirubinogen	5.04 mg.	1.126 mg.	1.251 mg.	1.17 mg.	2.455 mg.
Amount of urine	100 cc.	10 cc.	10 cc.	20 cc.	20 cc.
Amount of 20 per cent $FeSO_4 \cdot 7H_2O$	25 cc.	10 cc.	10 cc.	5 cc.	5 cc.
Amount of 10 per cent NaOH	25 cc.	10 cc.	10 cc.	5 cc.	5 cc.
Amount of filtrate used	2 cc.	2 cc.	2 cc.	6–5 cc.	5–5 cc.
Volume of the final solution	18 cc.	40 cc.	50 cc.	100–75 cc.	100–100 cc.
Percentage of P.S.P. standard	38	20	18	30–30	38–42
Amount recovered	3.69 mg.	0.86 mg.	0.97 mg.	1.07–0.972 mg.	1.64–1.83 mg.
Percentage recovered	73	76	77	87 (avg.)	70 (avg.)

* Crystalline urobilinogen isolated from urine.

stone. One portion served as a control, and to the other was added a known amount of crystalline urobilinogen.

1. Control. Five grams of the well mixed feces were subjected to the routine quantitative procedure described above except that half of the usual amounts of ferrous sulphate and sodium hydroxide solutions were employed, corresponding to 5 grams of feces, rather than 10. Forty cubic centimeters of the filtrate were subjected to the colorimetric procedure. The final solution measured 30 cc.

$$\frac{250}{5} \times \frac{30}{40} \times 0.13 \times 0.72 \times 0.05 = 0.175 \text{ mg. in 5 grams}$$

The result of a second determination in the same filtrate was:

$$\frac{250}{5} \times \frac{30}{40} \times 0.11 \times 0.72 \times 0.05 - 0.149 \text{ mg. in 5 grams}$$

Average: 0.162

2. After addition of crystalline urobilinogen. 2.77 mg. of crystalline urobilinogen were ground together with 5 grams of the same sample of feces and a quantitative determination made in the same way. 5 cc. of the filtrate were used, and the final colored solution measured 40 cc.

$$\frac{250}{5} \times \frac{40}{5} \times 0.18 \times 0.72 \times 0.05 = 2.59 \text{ mg.}$$

After subtraction of the amount already present in 5 grams, namely, 0.162 mg., the amount recovered is 2.42 mg. or 87 per cent. A second determination on the same filtrate:

$$\frac{250}{5} \times \frac{40}{5} \times 0.16 \times 0.72 \times 0.05 = 2.3 \text{ mg.} - 0.162 \text{ mg.} = 2.14 \text{ mg. or 77 per cent}$$

The average percentage recovered is 82 per cent.

On the basis of the above experiments it is evident that the range of recovery of urobilinogen with the quantitative procedure varies between 70 to 86 per cent for both urine and feces. For this reason the factor 0.72, the derivation of which has already been mentioned, has been omitted in routine calculations. As a result of this it is obvious that only approximate values can be obtained, but it is felt that greater accuracy probably cannot be attained for a substance as easily changed as is urobilinogen. If the above determinations are summarized, omitting the factor 0.72 in the calculation of each, it is seen that the agreement between the amounts added and amounts recovered is quite satisfactory (see table 3).

From this summary it would appear that omission of the factor 0.72 results in values slightly too high; however, smaller additional sources of loss undoubtedly

compensate in turn for this tendency. There is no doubt that small amounts of urobilin and mesobiliviolin are formed during the period of collection of the urine and feces even if optimal conditions for protection of the urobilinogen are maintained; it has already been seen that reduction of mesobiliviolin is not productive of a correspondingly intense Ehrlich reaction, and in the following it is seen that the reduction of urobilin is interfered with to some extent in the presence of urine.

(b) Recovery of crystalline stercobilin (urobilin) in terms of urobilinogen with the quantitative method.

1. Feces. For this determination acholic feces from a patient having a carcinoma of the head of the pancreas was used. The quantitative procedure had shown that as much as 50 cc. of filtrate from this feces yielded only negligible traces of urobilinogen.

TABLE 3

SPECIMEN	AMOUNT ADDED	AMOUNT RECOVERED
	mg.	*mg.*
Urine	5.040	5.13
	1.126	1.20
	1.251	1.35
	1.17	1.35
		1.49
	2.455	2.28
		2.54
Feces	2.77	2.98
		3.37

7.7 mg. of the crystalline hydrochloride of stercobilin were ground together with 10 grams of this feces. The quantitative procedure was then carried out, the primary dilution being 30. After standing over night 4 cc. of the colorless filtrate were subjected to the routine procedure. The final solution measured 32 cc., and was found equal to 30 per cent on the P.S.P. scale.

$$30 \times \frac{32}{4} \times 0.30 \times 0.72 = 72 \text{ mg. per 100 grams, or a recovery of 5.58 mg. (72 per cent)}$$

2. Urine. (a) Five milligrams of the crystalline hydrochloride were added to 50 cc. of urine found to contain negligible traces of urobilin or urobilinogen. This was mixed in a flask with 25 cc. each of 20 per cent $FeSO_4$ and 10 per cent NaOH. After standing over night 5 cc. of the filtrate were subjected to the

routine procedure. The final solution measured 50 cc. and was found of equal intensity to 38 per cent on the P.S.P. scale.

$$\frac{100}{50} \times \frac{50}{5} \times 0.38 \times 0.72 - 7.6 \text{ mg. per 100 cc., or a recovery of 2.84 mg. (57 per cent)}$$

(b) Another sample of urine was found to contain 0.024 mg. urobilinogen in 50 cc. by the quantitative method. 1.717 mg. of crystalline stercobilin hydrochloride were added to another 50 cc. portion. The result now obtained with the quantitative procedure, after subtraction of the 0.024 mg. originally present, was 1.106 mg. (in 50 cc.), or a recovery of 64 per cent.

(c) A similar determination gave the following results:

Control: 0.086 mg. in 75 cc. urine

After addition of 1.92 mg., the quantitative procedure found 1.260 mg.

Subtracting 0.086 mg. the amount recovered was 1.174 mg., or 61 per cent.

The lower percentage of recovery indicated by these results shows that the reduction of urobilin to urobilinogen is in some degree hindered in the urine.

III. Comparison of values obtained with the routine quantitative procedure

(a) A determination was made on each freshly passed stool during a four day period, and

(b) A determination was made on the mixed four day feces for the same period.

(a) 1. 178.7 grams feces contained 403.4 mg. urobilinogen
2. 137.8 grams feces contained 205.5 mg. urobilinogen
3. 124.2 grams feces contained 108.5 mg. urobilinogen
4. 27.0 grams feces contained 40.5 mg. urobilinogen

467.7 grams feces contained 757.9 mg. urobilinogen

During these 4 days 757.9 mg. of urobilinogen were excreted, or an average of 189.4 mg. per day.

(b) Since in each of the 4 daily determinations 10 grams of feces were used, a correction had to be made for the urobilinogen lost with these amounts. Thus 10 grams from (1) contained 22.5 mg. of urobilinogen, 10 grams from (2) 14.9 mg., 10 grams from (3) 8.7 mg., and 10 grams from (4) 15.0 mg. This made a total of 61.1 which had been removed in the 40 grams used for the above determinations. Accordingly the amount which could be expected was 174.2 mg. per day rather than 189.4 mg. The value found was 162.0 mg., or a 7 per cent decrease during the four day period. While the collection was in progress the feces were kept in a closed container at about 4°C. In this way error due to evaporation was largely eliminated.

IV. Use of P.S.P. colorimeter with permanent glass standards

The close similarity in color between the phenolphthalein standard of Terwen,[21] and an alkaline phenolsulphonphthalein solution suggested the use of

the P.S.P. colorimeter. The color comparison with the urobilinogen aldehyde solution in this apparatus has been found to be entirely satisfactory. If the final solutions are diluted so that their color intensity is in the range of 10 to 50 per cent of the P.S.P. scale, readings within 5 per cent are readily obtained. It has been customary to estimate values within the 5 per cent limits of the scale; for instance, if the color match is between 25 and 30, but obviously a little nearer 25, 27 per cent would be used in the calculation. Omission of the factor 0.72 in the final calculation has already been mentioned. Comparable values may be obtained with Terwen's alkaline phenolphthalein standard solution in another type of colorimeter if the final result is multiplied by 0.5.

V. Substitution of petroleum ether for ether

Ether was previously used in extracting urobilinogen from the acidified filtrates. Further experience with the method indicated that following ether extraction, the urobilinogen not infrequently underwent oxidation, causing interference with the color comparison, as well as a lowered value. This was particularly true in determinations of small amounts of urine urobilinogen where large amounts of filtrate were necessary. In such instances change occurred at times even when the most carefully purified ether was used, and it seemed probable that substances in the urine hastened the formation of peroxides in the ether. For this reason the use of ether was abandoned and petroleum ether is now used exclusively. The use of petroleum ether for the extraction of urobilinogen was mentioned by Terwen, although the routine procedure which he described made use of purified ether. Petroleum ether has never been noted to cause oxidation of urobilinogen, and has the further advantages of being easy to purify, and of being stable. Emulsions are not formed as frequently as with ether, and are usually readily separated with a few drops of alcohol. Occasionally with urines from patients having high grade obstructive jaundice, bad emulsions have occurred, and reduced values have undoubtedly been obtained on account of them, although this occurrence is fortunately rare.

Three extractions of the acidified filtrate with petroleum ether are necessary. A filtrate from the feces of a case of familial hemolytic icterus gave a value of 929.2 mg. of urobilinogen per day. This was obtained after a single extraction with petroleum ether. After three extractions of the same amount of filtrate the value obtained was 1424.9 mg. per day. One extraction therefore removed but 65 per cent. Three extractions with petroleum ether have always sufficed to remove all of the demonstrable urobilinogen. Felix and Moebus[6] recently stated that higher values were obtained if, instead of the filtrate, the entire mixture including the ferrous hydroxide was acidified and extracted. This has not been confirmed* in the present investigation; the following determinations are illustrative:

* It should be noted that Felix and Moebus employed the method to extract urobilinogen from ground-up liver, and it is quite possible that adsorption on precipitated protein explains their finding.

	Filtrate	Entire mixture
(1)	208.0 mg. per day	56.0 mg. per day
(2)	482.0 mg. per day	419.8 mg. per day
(3)	3.4 mg. per day	No color developed

VI. Additional changes which improve the previously[24] described quantitative procedure.

(a) In accordance with Terwen's method, an ethereal solution of p-dimethylaminobenzaldehyde was previously[24] employed for the Ehrlich reaction. This was added to the ether extract which was then shaken with concentrated hydrochloric acid. It has been found advantageous to extract the urobilinogen from the petroleum ether solution with the modified Ehrlich's reagent directly. The

TABLE 4

EFFECTS OF PRESERVATIVES

ONE-TENTH OF EACH SPECIMEN VOIDED IN DARK BOTTLE CONTAINING 1 CC. OF A SATURATED ALCOHOLIC SOLUTION OF SALICYLIC ACID	ONE-TENTH OF EACH SPECIMEN VOIDED IN FLASK CONTAINING 25 CC. 20 PER CENT FERROUS SULPHATE AND 25 CC. 10 PER CENT NaOH	REMAINING EIGHT-TENTHS OF EACH SPECIMEN IN DARK BOTTLE CONTAINING 5 GRAMS $NaCO_3$ AND 100 CC. PETROLEUM ETHER
mg. in 24 hours	*mg. in 24 hours*	*mg. in 24 hours*
2.7	3.2	4.2
1.5	2.7	4.1
4.8	10.8	12.0
1.7	4.3	6.9
Trace	1.2	2.0
No color	Trace	Trace
No color	Trace	0.4
1.6	1.6	2.7

aldehyde retains its purity in hydrochloric acid but gradually becomes brown in ethereal solution. Reasons for avoiding the use of ether have already been mentioned.

(b) To urines containing appreciable amounts of bilirubin, calcium hydroxide was added in the earlier procedure because it was believed possible that bilirubin might in part become reduced to urobilinogen by the ferrous hydroxide. Many urines rich in bilirubin, obtained from patients with complete common duct obstruction, have now been subjected to the quantitative procedure without calcium hydroxide treatment, but nevertheless, with the expected result, that is, the presence of not more than traces of urobilinogen. For this reason preliminary treatment with calcium hydroxide has been abandoned.

(c) Some evidence was obtained in the earlier investigation to indicate that slightly higher values might result following supplementary reduction with sodium amalgam. Further experience has shown this to be of doubtful value when the urine and feces are collected in the manner described and the determi-

nations carried out at once. Amalgam reduction has not been used in the present investigation.

(d) In accordance with Terwen, the earlier modification described by the writer[24] made use of salicylic acid to preserve the urine. In the present investigation it has been found that higher values are regularly obtained by other methods. Until recently one-tenth of each urine voided during 24 hours was at once placed in a flask containing the ferrous hydroxide mixture. This had the principal disadvantage of requiring careful cooperation on the part of the patient or attendants, and collections often had to be repeated because of inaccuracies. The only reason for mention of this method is that it was employed in a majority of the determinations which follow in parts II and III. Preservation by means of collecting the urine under petroleum ether, preventing extraction of urobilinogen at this stage by use of sodium carbonate, has been found to be both simpler and somewhat more efficient. The comparisons shown in table 4 made on the same 24-hour urines yield information as to the efficiency of these various methods of preservation.

SUMMARY AND CONCLUSIONS

A simplified procedure for the approximate quantitative estimation of the per diem amounts of urobilinogen in urine and feces is described. The color intensity of the Ehrlich reaction of crystalline mesobilirubinogen (urobilinogen) was found equal to that of crystalline stercobilin, or urobilin, after reduction with ferrous hydroxide. The colorimetric procedure was standardized with these crystalline substances. Recovery of crystalline urobilinogen added to samples of urine and feces varied from 70 to 87 per cent. Approximate allowance for this loss has been made in the quantitative estimation. Because urobilinogen is a labile chromogen, it is certain that no quantitative method can aspire to more than approximate values. It is pointed out that methods depending on urobilin characteristics cannot yield values corresponding to the original amounts of urobilinogen.

REFERENCES

(1) Adler, A., and Bressel, M. L.: Urobilinogen Bestimmung in Stuhl und Harn mittels der neuen Extraktionsmethode. Deutsch. Arch. f. klin. Med., **155**: 326–341. 1928.

(2) Bang, O.: Klinische Urobilinstudien insonderheit an normalen und "lebergesunden" Personen. Die Wirkung kohleydratknapper Kost auf die Urobilinausscheidung. Die Urobilinurie der Diabetiker. Acta med. Scandin. Supp., **29**: 1–197. 1929.

(3) ELMAN, R., AND MCMASTER, P. D.: Studies on urobilin physiology and pathology: IV. Urobilin and the damaged liver. Jour. Exper. Med., **42:** 99–122. 1925.

(4) ELMAN, R., AND MCMASTER, P. D.: Studies on urobilin physiology and pathology: V. Relations between urobilin and conditions involving increased red blood cell destruction. Jour. Exper. Med., **42:** 619–640. 1925.

(5) EPPINGER, H.: Die hepato-lienalen Erkrankungen. Berlin: J. Springer, 1920.

(6) FELIX, K., AND MOEBUS, H.: Das Verhalten von Urobilinogen in der Leber. Ztschr. f. physiol. Chem., **236:** 230–236. 1935.

(7) FISCHER, H.: Zur Kenntnis der Gallenfarbstoffe. I. Mitteil. Ztschr. f. physiol. Chem., **73:** 204–232. 1911.

(8) FISCHER, H., AND MEYER-BETZ, F.: Zur Kenntnis der Gallenfarbstoffe. II. Mitteil. Ueber das Urobilinogen des Urins und das Wesen der Ehrlichschen Aldehydreaktion. Ztschr. f. physiol. Chem., **75:** 232–239. 1911.

(9) FISCHER, H., AND NIEMANN, G.: Zur Kenntnis des Gallenfarbstoffs. VIII. Mitteil. Mesobiliviolin, Mesobiliviolinogen, und die Kondensation von Mesobilirubinogen mit Aldehyden unter Bildung von neuen Spaltprodukten. Diazofarbstoff des Mesobilirubins. Ztschr. f. physiol. Chem., **137:** 293–316. 1924.

(10) FISCHLER, F.: Physiologie u. Pathologie der Leber. 2 Aufl., Berlin: J. Springer, 1925.

(11) FISCHLER, F.: Ueber Frühdiagnosen der Lebererkrankungen. Deutsch. med. Wchnschr., 1167–1185. 1934.

(12) HEILMEYER, L., AND KREBS, W.: Ueber krystallisiertes Urobilin. Ztschr. f. physiol. Chem., **228:** 33–49. 1934.

(13) LICHTENSTEIN, A., AND TERWEN, A. J.: Ueber Blutmauserung und Urobilinausscheidung. Deutsch. Arch. f. klin. Med., **149:** 102–112. 1925.

(14) MCMASTER, P. D., AND ELMAN, R.: Studies on urobilin physiology and pathology: II. The derivation of urobilin. Jour. Exper. Med., **41:** 513–534. 1925.

(15) MCMASTER, P. D., AND ELMAN, R.: Studies on urobilin physiology and pathology: III. The absorption of pigments of biliary derivation from the intestine. Jour. Exper. Med., **41:** 719–738. 1925.

(16) MCMASTER, P. D., AND ELMAN, R.: Studies on urobilin physiology and pathology: VI. The relation of biliary infections to the genesis and excretion of urobilin. Jour. Exper. Med., **43:** 753–773. 1926.

(17) V. MÜLLER, F.: Ueber Ikterus. Jahresbericht d. Schlesische Gesellsch. f. vaterl. Kult. Breslau: Med. Sekt., 1–5. 1892.

(18) ROYER, M.: L'urobiline a l'etat normal et pathologique. Paris: Masson et Cie., 1930.

(19) ROYER, M.: Physiologie und Klinik des Urobilins. Klin. Wchnschr., **14:** 347–351. 1935.

(20) SAILLET: De la urobiline dans les urines normal. Rev. de med., **17:** 109–129. 1897.

(21) SINGER, K.: Studien zum Problem der Blutmauserung. I. Mitteil. Ueber den Einfluss der Ernährung auf die Urobilinogenausscheidung mit den Fäzes. Wien. Arch. f. inn. Med., **20:** 59–80. 1930.

(22) TERWEN, A. J. L.: Ueber ein neues Verfahren zur quantitativen Urobilin Bestimmung im Harn und Stuhl. Deutsch. Arch. f. klin. Med., **149:** 72–101. 1925.

(23) WALLACE, C. B., AND DIAMOND, J. S.: Significance of urobilinogen in the urine as a test for liver function. Arch. Int. Med., **35:** 698–725. 1925.

(24) WATSON, C. J.: The average daily elimination of urobilinogen in health and in disease, with special reference to pernicious anemia. Arch. Int. Med., **47:** 698–726. 1931.

(25) WATSON, C. J.: Ueber Stercobilin und Porphyrine aus Kot. Ztschr. f. physiol. Chem., **204:** 57–67. 1932.

(26) WATSON, C. J.: Stercobilin, Kopromesobiliviolin, Kopronigrin. Ztschr. f. physiol. Chem., **208:** 101–119. 1932.

(27) WATSON, C. J.: Isolation of crystalline urobilin from human urine. Proc. Soc. Exper. Biol. and Med., **30:** 1210–1211. 1933.

(28) WATSON, C. J.: Ueber krystallisiertes Harn-Urobilin sowie weiteres ueber Stercobilin und Kopromesobiliviolin. Ztschr. f. physiol. Chem., **221:** 145–155. 1933.

(29) WATSON, C. J.: Isolation of mesobiliviolin from human feces; its origin and nature. Proc. Soc. Exper. Med. and Biol., **30:** 1207–1209. 1933.

(30) WATSON, C. J.: An improved method for the isolation of crystalline stercobilin. Jour. Biol. Chem., **105:** 469–472. 1934.

(31) WATSON, C. J.: A crystalline iron chloride molecular compound of urobilin and stercobilin. Proc. Soc. Exper. Biol. and Med., **32:** 534–536. 1934.

(32) WATSON, C. J.: Ueber krystallisiertes Stercobilin bzw. Urobilin. IV. Mitteil. Ztschr. f. physiol. Chem., **233:** 39–58. 1935.

(33) WATSON, C. J.: The origin of natural crystalline urobilin (stercobilin). Jour. Biol. Chem., **114:** 47–57. 1936.

(34) WEISS, M.: Ueber Urobilin und seine diagnostische Verwertung. Wien. Arch. f. inn. Med., **20:** 39–58. 1930.

(35) WILBUR, R. L., AND ADDIS, T.: Urobilin: Its clinical significance. Arch. Int. Med., **13:** 235–286. 1914.

A STUDY OF THE ORGANISMS AND SERUMS FROM A NUMBER OF TYPHOID PATIENTS AND CARRIERS*

LOIS ALMON AND W. D. STOVALL

State Laboratory of Hygiene, Madison, Wisconsin

The possible implications of the discovery of the recently reported "Vi" antigen are many. (Felix and Pitt.[3]) The presence of the antigen in cultures of the typhoid organism is reported to be associated with virulence. It might be expected, then, that cases from which organisms containing the "Vi" antigen were isolated would be more severe than those from which organisms containing none of it were isolated. It might be expected also that the progress of the disease would be affected by the presence or absence of the corresponding antibody in the patients' serums. Then too, it is conceivable that the type of organism and the presence or absence of certain antibodies might be correlated with the carrier state. These considerations are the subject of the researches reported here.

Somewhat similar studies have been made by Felix, Krikorian, and Reitler.[2] They studied ninety freshly isolated cultures for the presence of the "Vi" antigen and found it in eighty-eight of them. They report, however, that none of these strains was of the extremely virulent type; that all of them which contained the "Vi" antigen fell into the group of intermediate virulence. In the study of the serums of 117 patients and one carrier, the "Vi" antibody was found in only thirteen of the patients and in the carrier; and in those serums in which it was found, the titer was sometimes no higher than 1:10. Moreover, it was found in a higher percentage of the serums from convalescents than of those taken from patients during the pyrexial stage. These authors

* Read before the Fifteenth Annual Convention of the American Society of Clinical Pathologists, held at Kansas City, Missouri, June 8 to 10, 1936.

TABLE 1

CLASSIFICATION OF CASES

PATIENT	DURATION OF HIGH TEMPERATURE	HIGHEST TEMPERATURE RECORDED	DURATION OF FALLING TEMPERATURE	BLOOD ON ENTRANCE	DIARRHEA	COMPLICATIONS OR SPECIAL SYMPTOMS	IN HOSPITAL
				Mild to moderate cases			
	days		*days*				*days*
1	21	102.4	7	No record	−	None	28
2	19	103	7	Hb. 80% Rbc. 4,400,000 Wbc. 4,550	+	None	39
3	8	104	5	Hb. 85% Rbc. 3,980,000 Wbc. 8,550	−	None	28
4	8	103.2	4	Hb. 78% Rbc. 4,300,000 Wbc. 4,800	−	None	27
5	22	103	16	Hb. 70% Rbc. 3,940,000 Wbc. 6,700	−	None	43
6	18	104.2	17	Hb. 75% Rbc. 4,470,000 Wbc. 7,000	−	1 mild relapse	82
7	25	104.4	8	Hb. 75% Rbc. 4,490,000 Wbc. 4,500	−	None	26
8	14	104.4	6	No record	−	None	44
				Severe cases			
9	12	104	10	Hb. 80% Rbc. 4,240,000 Wbc. 5,800	−	Thrombo-phlebitis 53 days	85
10	7	104.5	−	Hb. 90% Rbc. 4,380,000 Wbc. 7,100	+	Hemorrhage. Died after 7 days	7

TABLE 1—*Concluded*

PATIENT	DURATION OF HIGH TEMPERATURE	HIGHEST TEMPERATURE RECORDED	DURATION OF FALLING TEMPERATURE	BLOOD ON ENTRANCE	DIARRHEA	COMPLICATIONS OR SPECIAL SYMPTOMS	IN HOSPITAL
			Severe cases—*Continued*				
	days		*days*				*days*
11	25	104	27	No record	No record	Irrational	66
12	23	105	25	Hb. 80% Rbc. 4,750,000 Wbc. 7,400	+	Intestinal hemorrhage 11th day. Rbc. down to 2,620,000; Wbc. 3,500; Thrombo-phlebitis 36th day	73
13	32	106.6	No record	No record	No record	"Very toxic"	34 Convalescent at home

state that owing to the failure to isolate any strains of organisms of the extremely virulent type, "no attempt could be made to investigate whether or not there is any correlation between the severity of the disease and the type of strain isolated from the patient." Nevertheless, they describe wide variations in the cases from which they isolated organisms classified by them as intermediate in virulence, such terms as "mild," "moderately severe," "very severe," and "extremely severe" being used in their table 5 with reference to these cases.

Kauffmann,[4] reported that all strains recently isolated from cases contained the "Vi" antigen, although occasional colonies lacking it occurred on some of the plates.

CASES STUDIED

Our plan was to classify the active cases in our survey into two groups: severe, and mild to moderate; based upon the height

of the fever, the symptoms of toxicity, and the occurrence of complications. Carriers formed an additional class. Most of these cases occurred in local hospitals where the histories were readily accessible.

A summary of the salient features of these cases is contained in table 1. Of the four carriers (see table 2) from which cultures were obtained we have histories of only two. Patient 14 had had a case of typhoid of moderate severity two years previous to his readmission to the hospital for removal of his gall bladder, at which time our culture was isolated. Patient 15 had had typhoid fever six years previous to the time her carrier state was discovered. Cultures of typhoid organisms could still be isolated from her feces even after cholecystectomy. Our culture was isolated from the gall bladder, at the time of its removal.

COMPARISON OF THE "VI" CONTENT AND VIRULENCE OF CULTURES WITH THE CHARACTER OF THE CASES FROM WHICH THEY WERE ISOLATED

The cultures isolated from the various cases were classified as to the presence or absence of the "Vi" antigen. This was done by two methods. The first was to test their agglutinability in a pure "Vi" antiserum; that is, a serum produced by injecting rabbits with live suspensions of a culture known to possess the antigen, culture Ty 2 of Felix in this instance, and absorbing the resulting antiserum with suspensions of a culture, H 901, which possessed none of the antigen. The cultures which agglutinated in this serum were assumed to contain the "Vi" antigen. The second method was used as a check in a few instances, since it had been shown as a result of previous work (Almon and Stovall,*) that the failure of cultures to agglutinate in this serum did not preclude the possibility of their containing the antigen. This method was to prepare antiserums with the cultures and perform absorptions on these, again with culture H 901, in order to determine whether "Vi" antibodies were formed. The results of these two methods of determining the "Vi" content of the cultures are reported in table 2 in the columns headed

* Unpublished data.

"Agglutination titer in 'Vi' antiserum," and "'Vi' titer of homologous antiserum," respectively.

In the virulence tests twenty mice were injected intra-peritoneally with one hundred million organisms contained in 0.5 cc. of saline solution. The number of mice which died within a 48 hour period were then counted. These results also appear in table 2.

Two things are shown in this table. One is that organisms containing the "Vi" antigen as well as organisms lacking it may

TABLE 2

"VI" CONTENT AND VIRULENCE OF ORGANISMS ISOLATED FROM CASES STUDIED

	CASE	ORGANISMS ISOLATED	AGGLUTINATION TITER IN "VI" ANTISERUM	"VI" TITER OF HOMOLOGOUS ANTISERUM	RATIO: MICE KILLED / MICE INJECTED
Mild to moderate	3	T 4	5120	320	7/20
	4	T 3	0	0	4/20
	6	T 7	0	Not done	6/20
	7	T 10	5120	Not done	6/20
Severe	10	T 2	5120	1280	2/20
	11	T 5	0	0	6/20
	12	T 18	2560	Not done	3/20
	13	T 9	0	Not done	5/20
Carriers	14	T 6	5120	320	9/20
	15	T 17	5120	Not done	4/20
	16	T 8	5120	Not done	6/20
	17	T 19	0	0	1/20

be isolated from cases of varying degrees of severity and from carriers. Evidence from both agglutination in "Vi" serum and production of "Vi" antibodies in rabbits indicated that both types of cultures had been isolated from cases reacting with different degrees of severity. The other thing is that there is no positive correlation either between the virulence of a given culture for mice and its "Vi" content or between virulence for mice and the severity of the case from which it was isolated. Culture T 5, which showed no evidence of the presence of "Vi" antigen by any test, killed as many mice as did most of the cultures which

possessed the antigen and more than some of them. Also, culture T 2, isolated from patient 9, who died on his seventh day in the hospital, killed fewer mice than did most of the cultures obtained from the milder cases.

An additional conclusion of interest may be drawn from these data; that is, that the organisms from various cases in an epidemic, presumably of common origin, may differ in their antigenic constitution. Patients 3, 4, 10 and 11, all occurred in an epidemic among a circus troupe. The cultures from two of these cases showed evidence of the presence of the "Vi" antigen, whereas the other two did not.

VARIATIONS IN "VI" CONTENT AMONG SUB-CULTURES FROM ORIGINAL ISOLATIONS

Variations in the "Vi" content of cultures isolated from several cases during an epidemic of single origin may be more easily understood when it is known that the individual cells making up a given culture may show similar variations.

Three single cells were isolated by the modified Chambers micromanipulator from culture T 6. The cultures resulting from their multiplication were carefully checked for motility, fermentation characteristics, and agglutination in anti-typhoid sera to establish their identity as typhoid organisms. They were then tested for their agglutinability in pure "Vi" antiserum as an approximate measure of their "Vi" content. It was found that two of the cultures agglutinated through a dilution of 1:5120 of this serum, as had the stock, T 6, from which they had been derived. The other single cell culture, however, failed entirely to agglutinate in this serum, indicating that it lacked or possessed only small amounts of the "Vi" antigen.

These results were repeated with sub-cultures from stock, T 2, obtained by plating and picking well isolated colonies. Since organisms from typhoid patients are isolated in diagnostic laboratories by picking colonies from plates, the kind of culture with respect to its "Vi" content isolated from a given case may not be a fair indication of the antigenic constitution of the organisms predominating in the body. Since these isolations are subject

to the laws of chance, there is greater opportunity, however, of isolating a typical culture than of isolating one which is representative of the minority of organisms causing the infection.

The knowledge of the antigenic differences among the individuals of the bacterial population in a culture helps to explain the fluctuations which Felix and his associates as well as we have observed in the "Vi" content of cultures of the "intermediate" type. Kauffmann, is in accord on this point.

"O," "H," AND "VI" ANTIBODIES IN PATIENTS' SERUMS

Much has been written concerning the antibodies produced during active typhoid infection. It is generally believed, for example, that the "O" antibody appears early in the disease, and that it is this factor which is of prime importance in combating the infection. Since the discovery of the "Vi" antigen, it has been claimed by Felix,[1] that its antibody is also important in hastening recovery, and antiserums containing it have been administered therapeutically.

The study from which the results in table 3 have been derived was an attempt to discover whether a mild or moderate clinical picture could in any way be correlated with the kind or titer of the serum antibodies in the case concerned.

For this purpose the serum was titrated when possible against the organisms which had been isolated from the case, and also against a standard alcoholic suspension for the "O" titer, and a standard formalinized suspension for the "H" titer. For the "Vi" titer, the serum was absorbed with culture H 901 of Felix and then tested against a culture Ty 2 of Felix for remaining "Vi" antibodies. This titration was started with a dilution of 1:40, for absorption complete enough to make possible accurate readings in lower dilutions was difficult to attain.

Again the results are noteworthy for their lack of correlation with anything in the clinical history. It is true that the two cases which developed "Vi" antibodies occurred in the mild to moderate group. But not enough severe cases have been studied to prove that these do not also sometimes develop the antibodies. Since the "Vi" antibody was not present at any time in the sera

TABLE 3

"O", "H", AND "Vi" TITERS OF PATIENTS' AND CARRIERS' SERUMS

CASE	"VI" PRESENT IN ORGANISMS ISOLATED	ILLNESS	TITER FOR LIVE HOMOLOGOUS ORGANISMS	"O" TITER	"H" TITER	"VI" TITER
		Mild to moderate				
		days				
1	No organism isolated	22		1280	200	0
	No organism isolated	37		2560	640	0
	No organism isolated	59		2560	1280	0
2	No organism isolated	4		2560	0	0
	No organism isolated	19		2560	0	0
	No organism isolated	41		2560	160	0
3	+	6	2560	2560	5120	0
	+	21	5120	5120	5120	0
5	No organism isolated	45		640	320	40
6	−	8	1280	1280	0	0
	−	17	2560	2560	160	0
	−	29	5120	5120	320	0
7	+	30	2560	2560	2560	1280
	+	42	2560	2560	2560	640
8	No organism isolated	18		1280	320	0
		Severe				
9	No organism isolated	7		2560	640	0
	No organism isolated	44		5120	1280	0
10	+	9	640	640	320	0
11	−	18	5120	5120	1280	0
	−	40	2560	2560	640	0
12	+	31	10240	10240	2560	0
		Carriers				
15	+	6 yrs.	2560	0	2560	40
17	−	Not	640	0	1280	0
	−	known	2560	640	2560	0

of those patients from whom we had samples covering the entire period of illness, we are unable to say how early it develops. In the two instances in which we demonstrated it in active cases, the blood was drawn late in the clinical course. One thing of interest is that patient 7, who showed the highest "Vi" titer of any in the series, is known to have contracted his infection from carrier 15, who also developed "Vi" antibodies. This small amount of evidence is suggestive of the possibility that certain strains of the species are better able than others to stimulate the production of the "Vi" antibodies in human hosts, even though these strains differ in no way by laboratory tests from others which fail to stimulate "Vi" antibody production in patients. Another instance among these data of similarity between organisms and antibodies in two cases, one of which is known to be the result of infection from the other, occurs in cases 6 and 9. The former was infected with organisms from the latter. In neither case were "Vi" antibodies present in the sera.

The "O" and the "H" antibodies seem to yield a more definite story. With three exceptions, cases 3, 7, and 11, among the active cases, the "O" titer developed to a higher point than did the "H" titer. In only one of the exceptions, case 3, did the "H" titer exceed the "O" at any period of the disease.

In the cases of the two carriers from whom we were able to obtain serums, the "O" titer was low or lacking, but the "H" titer was high.

None of the serums tested failed to agglutinate live organisms. Moreover, the titer for the live organisms was in every case as high as for any other type of suspension. This may be of importance in laboratory diagnosis.

DISCUSSION AND SUMMARY

Whether the "Vi" antigen plays a part in determining the virulence of typhoid organisms is difficult to ascertain because the amount of the antigen in a given culture can not be stabilized and measured. The ratio within a given bacterial population of organisms which have it to organisms which do not, fluctuates with each transfer and each new isolation. By means of the

methods at present at our disposal, certainly no correlation has been found between the "Vi" content of a given culture and its virulence either for mice or for the host from which it was isolated.

The "Vi" antibodies can be more accurately studied, if cultures are at hand which are known to lack "Vi" antigen and can be used for absorption and if others are at hand which are known to be relatively stable in their "Vi" content and can be used in agglutinations as indicators of the antibodies. Cultures H 901 and Ty 2, respectively which we obtained from Felix serve very well for these purposes. By the use of these cultures the "Vi" antibody may be demonstrated in the sera of some patients and carriers. But the rôle of the antibody in the clinical course of the case remains obscure for want of more abundant data.

A survey of the "O" and "H" antibodies in the patients' serums revealed nothing new. The "O" antibody was shown to develop first, and to higher titer than the "H," in most cases. In the carriers the "H" was higher.

Incidental to the information summarized above, we gleaned a certain number of suggestions which may be of value in future investigations and in routine laboratory work. In condensed version, these may be set forth thus:

1. Live suspensions of typhoid organisms usually agglutinate primarily in compact granular clumps. In human serums, the clumps are apt to be smaller than in rabbit immune serums.

2. The selection of a non-motile strain of the typhoid organism for the preparation of "O" suspensions for routine Widal tests may not be the best procedure. The non-motile cultures in our collection have shown eccentricities in agglutinability which should make their use as detectors of "O" antibodies questionable. Motile strains exist which make superior "O" antibody detectors. The same motile strains may be used to prepare "H" suspensions.

3. A special "Vi" suspension need not be used in Widal tests since "Vi" antibodies occur so infrequently, in such low titer, and have never been found to be the sole type of antibody present in patients' serums.

4. If anti-serums containing "Vi" in addition to "O" and "H" antibodies are used in the identification of organisms sus-

pected of being *Eb. typhosa*, there need be no fear of "inagglutinability" complicating the result.

REFERENCES

(1) FELIX, A.: Clinical trials with a new antityphoid serum. Lancet, **1**: 799–802. 1935.

(2) FELIX, A., KRIKORIAN, K. S., AND REITLER, R.: The occurrence of typhoid bacilli containing Vi antigen in cases of typhoid fever and of Vi antibody in their sera. Jour. Hyg., **35**: 421–427. 1935.

(3) FELIX, A., AND PITT, R. M.: A new antigen of B. typhosus. Lancet, **1**: 799–802. 1935.

(4) KAUFFMANN, F.: Ueber einen neuen serologischen Formenwechsel der Typhusbacillen. Ztschr. f. Hyg. u. Infectionskrankh., **116**: 617–651. 1935.

ERYTHROCYTE COUNTS, HEMOGLOBIN, AND ERYTHROCYTE VOLUME IN NORMAL YOUNG MEN AND WOMEN RESIDING IN THE EASTERN UNITED STATES*

WILLIAM P. BELK†

Clinical Biochemistry, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

ELSIE CURTIS†

Clinical Laboratory, Hospital of the Woman's Medical College of Pennsylvania, Philadelphia, Pennsylvania

AND

MARGARET K. WILSON†

The value of erythrocyte size and hemoglobin concentration in the clinical study of anemia has long since been thoroughly demonstrated. These data supply information, not available by other means, concerning the type of an anemia, its possible etiology, the most appropriate therapy, (whether iron or liver) and the ultimate adequacy of therapy. This method, however, has not come into such general use as its value merits. Although, as a practical aid in the study of anemia, it may be fairly compared to the nuclear shift in the study of infections, it is much less commonly employed. The reason for this is doubtless the difficulty of selecting suitable normal standards. As a consequence, the calculation of volume, saturation and color indexes frequently gives discrepant and even impossible results, which discourage further use of the method.

The present study was undertaken in the belief that (for us) the difficulty of standards could be overcome by determining our own standards of normal. It appeared logical that any peculiari-

* Read before the Fifteenth Annual Convention of the American Society of Clinical Pathologists, held at Kansas City, Missouri, June 8 to 10, 1936.

† Times Medical Building, Ardmore, Pennsylvania.

ties of technic, which might render our results unsuitable for comparison with the normal values of other workers, would be incorporated in our standards, and that these, therefore, would be most appropriate for the study of our patients. In addition, we wished to add to the data previously compiled of the number of erythrocytes, hemoglobin and volume of packed erythrocytes in normal young men and women, and to determine whether or not there is any peculiarity in these values attributable to geographic location in the Eastern United States, no such study having been made in this locality.

The subjects were students in Villa Nova College, near Philadelphia, and nurses and technicians in the Hospital of the Woman's Medical College of Pennsylvania, in Philadelphia. All stated that they had resided in the Eastern United States for at least three years, and most for the duration of their lives. One man had spent the previous school year in Oregon. All were carefully questioned concerning the state of their health, and the majority had had a satisfactory physical examination within six months. The men averaged eleven pounds above the expected weight for age and height. About half of them were athletes, although none were in training at the time examined. The women averaged three pounds above the expected weight for age and height. Blood was collected from the men between 9.00 and 9.30 a.m., and from the women between 10.00 a.m. and noon. None of the subjects had taken more than very light exercise for some hours before being bled. In the case of the women samples were collected at least one week after the last menstrual period. Racially the men represented a varied group; the women were practically all of old American stock. The elevation of Villa Nova above sea level is 465 feet, that of the Hospital of the Woman's Medical College 210 feet.

TECHNIC

All determinations were made on 5-cc. amounts of venous blood collected with a minimum of stasis and rendered incoagulable with 10 mg. of dry potassium oxalate. The tubes were kept stoppered to prevent evaporation, and were thoroughly shaken just prior to the removal of each sample. All studies were completed within 9 hours of the collection of the specimens.

Erythrocyte counts were made with Thoma pipets and the Levy-Hausser counting chamber bearing the Bureau of Standards certificate. The diluent was normal saline made from Reagent grade sodium chloride. Pipets, after being filled, were shaken vigorously by hand in a direction transverse to their long axis for 3 minutes, and at least one-third of their contents was expelled before filling the chamber. The counts recorded are the average of two which agreed within 200,000 cells. When counts did not so agree, the chamber was refilled from the same two suspensions. If they again did not agree, the counts were repeated from the beginning. We realize, however, that, in our hands, such close agreement was a matter of chance, as the standard error of the mean of our two counts was found to be 0.21 millions. However, the limitation to two consecutive counts differing by not more than 0.2 very greatly reduced the standard error of the estimated mean. This practice of repeating pairs of counts by refilling the chamber with the original suspensions was justified by the following observation:—Two series of five complete counts were made on a single specimen of blood, and from one pipet of each series the counting chamber was filled and counted five times. Statistical analysis showed no significant difference between the standard deviation of the completely and the partially duplicated series of counts. The error of filling the chamber was large compared with that of filling the pipets.

Hemoglobin determinations were made in duplicate with the Laboratory Model Haden-Hausser hemoglobinometer, the blood being diluted with carefully made N/10 hydrochloric acid in Thoma leukocyte diluting pipets bearing the certificate of the Bureau of Standards. Readings were made at least 30 minutes after dilution. In no instance was there a variation of more than 0.5 gram between the two observations, and we assume that the recorded figures for individuals are correct within 0.25 gram for this instrument. The instrument was not corrected by the van Slyke or other method. It was shown, however, to have a calibration error in that when readings were made on carefully prepared dilutions of a solution of acid hematin, the upper part of the scale gave values relatively lower by about 3 per cent than the lower part. Upon completing the study it was found that our averages for cell numbers and for volume of packed cells in both men and women agreed closely with those of a large number of observations collected by Wintrobe.[6] Our hemoglobin averages, however, were somewhat lower (96 per cent of Wintrobe's for men, 94 per cent of Wintrobe's for women). We felt justified, therefore, in assuming, because of this close correlation in two values, that the hemoglobinometer in the region of the normal values was calibrated at approximately 97 per cent of the true value. We consequently corrected our hemoglobin readings by dividing them by 0.965. The average value for men was thus increased by 0.55 gram, that for women by 0.37 gram. Even had this correction not been made, conclusions expressed later in the text would still be justified.

Packed erythrocyte volumes were determined with the Wintrobe hematocrit by recentrifuging to constant volume. It was noted that the time necessary to

effect complete packing varied somewhat with different samples. Wintrobe found this instrument accurate to 1 per cent. Preliminary observations, in which readings on individual samples were made in all our tubes, confirmed this figure. The results before recording were increased by 3.5 per cent to correct for the shrinkage caused by the oxalate.[4]

TABLE 1

OBSERVATIONS ON THE BLOOD OF TWENTY-FIVE NORMAL YOUNG MEN

SUBJECT	AGE	ERYTHROCYTES	HEMOGLOBIN	VOLUME OF CELLS	COLOR INDEX	VOLUME INDEX	SATURATION INDEX
		millions	*grams*	*cc.*			
1	23	5.23	15.50	46.6	0.99	1.04	0.95
2	22	4.99	15.25	46.1	1.03	1.07	0.96
3	20	5.11	15.00	42.6	0.99	0.98	1.01
4	21	5.56	15.50	45.5	0.95	0.95	0.99
5	21	5.57	16.90	51.7	1.03	1.08	0.95
6	23	5.58	15.50	43.5	0.94	0.90	1.04
7	23	5.41	15.50	44.0	0.97	0.94	1.03
8	22	5.46	16.60	46.1	1.02	0.98	1.04
9	22	5.13	17.40	49.7	1.04	1.10	1.03
10	21	5.27	16.60	46.6	1.07	1.03	1.04
11	17	5.44	15.50	45.6	0.96	0.97	0.99
12	21	4.63	15.00	43.5	1.09	1.08	1.00
13	18	5.42	16.60	46.1	1.04	0.99	1.05
14	18	5.45	15.50	47.6	0.96	1.02	0.95
15	23	5.73	17.15	49.7	1.00	1.01	0.99
16	23	5.02	16.05	47.1	1.08	1.10	0.98
17	19	4.90	16.60	46.1	1.14	1.09	1.05
18	23	5.82	15.50	44.6	0.91	0.89	1.02
19	18	5.29	15.50	47.6	0.99	1.04	0.95
20	19	5.34	15.50	43.0	0.98	0.93	1.05
21	20	5.34	17.15	47.6	1.07	1.04	1.04
22	18	6.02	15.00	45.5	0.84	0.88	0.96
23	20	5.70	16.05	45.0	0.95	0.92	1.03
24	21	6.04	16.35	48.1	0.91	0.92	0.98
25	22	4.85	14.75	45.0	1.03	1.08	0.95
Averages...	20.7	5.37	15.92	46.2	1.00	1.00	1.00
Coefficients.....		5.00	14.82	43.0			

The data of this study are given in tables 1 and 2. In table 3 our averages are compared with those compiled by Wintrobe from a large number of accurate determinations. The volume coefficients and the corpuscular constants in this table were calculated by us from Wintrobe's figures (p. 124).

The results of this study are seen to agree closely with those of the larger series. Our figures may be compared also with an average erythrocyte count of 5,000,000 determined by Emerson[2] for men living in Baltimore; with an average hemoglobin of 15.05 grams determined by Harrop[3] for men living in Balti-

TABLE 2

OBSERVATIONS ON THE BLOOD OF TWENTY-FIVE NORMAL YOUNG WOMEN

SUBJECT	AGE	ERYTHROCYTES	HEMOGLOBIN	VOLUME OF CELLS	COLOR INDEX	VOLUME INDEX	SATURATION INDEX
		millions	*grams*	*cc.*			
1	21	4.62	13.65	40.4	1.03	1.02	1.01
2	21	4.10	14.45	43.0	1.22	1.22	1.00
3	20	4.38	12.85	38.8	1.02	1.03	0.99
4	21	4.53	13.10	39.8	1.01	1.02	0.99
5	23	4.33	12.30	40.9	0.99	1.10	0.91
6	19	4.37	14.45	41.9	1.14	1.11	1.03
7	21	4.46	12.85	36.7	1.02	0.96	1.06
8	23	5.12	13.35	43.5	0.92	0.99	0.92
9	18	5.59	14.45	44.5	0.89	0.92	0.97
10	24	4.91	15.00	40.4	1.06	0.94	1.11
11	19	4.35	13.95	39.3	1.11	1.05	1.06
12	18	5.07	13.95	40.4	0.96	0.93	1.03
13	19	4.06	13.35	37.3	1.14	1.06	1.08
14	19	4.47	12.30	37.3	0.97	0.97	0.99
15	18	4.83	13.35	41.4	0.97	0.99	0.97
16	23	4.84	13.35	41.4	0.97	0.99	0.97
17	20	4.72	13.35	41.4	0.99	1.02	0.97
18	19	4.94	13.95	40.4	0.98	0.95	1.03
19	19	4.37	13.95	40.4	1.11	1.07	1.03
20	19	4.47	13.35	42.4	1.04	1.10	0.95
21	21	4.90	12.30	40.4	0.88	0.96	0.92
22	23	4.77	13.35	40.9	0.98	1.00	0.98
23	19	5.00	12.55	37.8	0.88	0.88	1.00
24	21	4.74	12.55	36.2	0.93	0.89	1.04
25	20	5.16	14.65	43.5	0.98	0.98	1.00
Averages...	20	4.70	13.47	40.4	0.97	1.01	1.00
Coefficients.....		5.00	14.33	43.0			

more; and with an average hemoglobin of 15.4 grams determined by Dill[1] for men living in Boston. These figures and ours are practically within the limits of technical variation of studies made in other parts of the United States. When the individuals in the present series are considered, all the men are seen to fall within the normal limits set by Wintrobe. Erythrocyte counts in two women,

and cell volume in two other women fall just without these limits. Since it is generally agreed that the differences in the average normal erythrocyte count, hemoglobin and packed cell volume, as determined in previous observations, are due to technical considerations rather than to geographical location, it may be assumed that the slight variations of this study are likewise technical, and that

TABLE 3

NORMAL ERYTHROCYTE VALUES

	MEN		WOMEN	
	Wintrobe	This study	Wintrobe	This study
Erythrocytes	5.40	5.37	4.85	4.70
Hemoglobin	16.00	15.92	13.90	13.47
Hemoglobin coefficient	14.80	14.82	14.30	14.33
Cell volume	46.30	46.20	41.40	40.40
Volume coefficient	42.87	43.00	42.68	43.00
Corpuscular volume	85.74	86.00	85.36	86.00
Corpuscular hemoglobin	29.63	29.64	28.66	28.66
Corpuscular hemoblogin concentration	34.56	34.46	33.57	33.34

TABLE 4

COMPARISON BETWEEN STANDARDS PROPOSED BY WALTERS AND BY WINTROBE AND MILLER[7]

(Walters' standards taken as 100 per cent of normal)

	WINTROBE AND MILLER	
	Average	Case 30 (normal case)
Volume index	0.82	0.71
Saturation index	1.12	1.09
Color index	0.92	0.77
Corpuscular volume	79.5 (82–107)	70
Corpuscular hemoglobin concentration	36.6 (27.5–36.5)	34.6
Corpuscular hemoglobin	29.1 (25–37)	24.2

Parentheses indicate normal ranges according to Walters.

no peculiarity in these values attributable to geographical location in the Eastern United States is shown.

COMMENT

Walters[5] called attention to the fact that no universally satisfactory standards for normal erythrocyte numbers, hemoglobin

and packed cell volume can be found, because of the unexplained (apparently technical) and considerable differences which appear when the results of different workers are compared. The chief error, as Haden has observed, is in the marked variation in erythrocyte counts, although variations in hemoglobin determinations are important. Estimations of the normal volume of packed cells agree closely. The nature of the calculations used in deter-

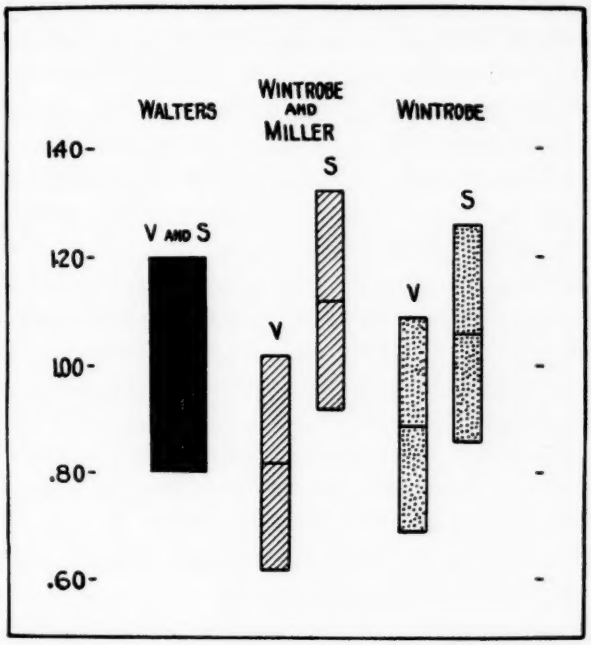


FIG. 1. DISTRIBUTION OF VOLUME INDEX AND OF SATURATION INDEX OF WINTROBE AND MILLER'S NORMAL SUBJECTS, AND OF WINTROBE'S COLLECTED NORMAL SUBJECTS FROM THE LITERATURE WHEN CALCULATED WITH WALTERS' STANDARDS OF NORMAL.

mining coefficients and indexes magnifies the differences in the basic values, often to a significant degree. Consequently, when the normal values recorded by one observer, or even the average normals collected by Wintrobe are used, by comparison, to determine the normality or abnormality of the blood of an individual patient, studied by a somewhat different technic, obviously incorrect results must of necessity be frequent. Table 4 and figure 1

illustrate how frequently volume and saturation indexes well without the normal zone may be found in individuals actually normal merely by the chance selection of an inappropriate standard for comparison. The suggestion that the use of the corpuscular constants would obviate the necessity for accurate normal standards hardly solves the difficulty, for these are not really constants. They vary with the different studies, as do the values from which they are calculated. The point of importance is this: if skilled workers differ so markedly, for technical reasons, in their determinations of numbers of erythrocytes and amount of hemoglobin, how much more must the average technician differ from these and all other normal standards? For this reason it would seem most advisable that each institution determine the normal erythrocyte values for its own peculiar technic, and use these for the calculation of percentages and indexes. Until this be done it is probable that the study of erythrocyte size and hemoglobin concentration, in spite of its great practical value, will not come into general use. For those unable to make such determinations Wintrobe's averages of a large number of observations are of course most applicable.

Our experience with the use of our own standards has been much more satisfactory than with the use of borrowed normal values. Results obviously in discord with the other clinical features of cases studied and unexplained abnormal values are now infrequent.

It has been suggested (as a compromise, not as an ideal arrangement) that an average of the hemoglobin coefficients for men and women (14.5) be used as 100 per cent hemoglobin in the manufacture of hemoglobinometers reading in per cent. When, however, this standard is applied to the average normal and normal range for hemoglobin, it is seen that the figures for men vary from 14 to 18 grams (97 to 124 per cent, average 110 per cent); and for women from 12 to 16 grams (83 to 110 per cent, average 97 per cent). The standard is correct for neither sex, being slightly too high for women, and definitely too low for men. In the clinical study of individual patients this would lead to confusion. Since there is a definite difference in the actual amount of hemoglobin

in men and women, it would seem more practical to employ a different value for 100 per cent in the two sexes. When an instrument reading in grams is used, such a comparison, either in grams or in per cent, is easy. Unfortunately, with instruments reading in per cent, one must either recalculate or readjust his concept or normal to some figure other than 100 per cent.

Wintrobe's hemoglobin coefficient for both men and women (14.5) can, however, be used for the calculation of the erythrocyte indexes, as the difference between results derived from this figure and from Wintrobe's individual coefficients for men and women is quite small. In table 3 it is seen that the volume coefficients for the two sexes is exactly the same in this study (43), and practically the same for Wintrobe's averages (42.87, men; 42.68, women). It seems justifiable, therefore, to take 43 as the volume coefficient for both men and women. Consequently, the calculation of the *relative* values, that is the color, volume, and saturation indexes, may be simplified by taking 5,000,000 cells, 14.5 grams hemoglobin, and 43 per cent volume of packed cells as 100 per cent in both sexes. This statement is not intended to contravene the opinion expressed above that individually determined standards are the most practicable.

CONCLUSIONS

No geographical peculiarity is demonstrated in erythrocyte numbers, hemoglobin, or packed erythrocyte volume of normal young men and women residing in the Eastern United States.

A hemoglobin coefficient of 14.5 (Wintrobe) is proposed for the calculation of color and saturation indexes (not for expressing hemoglobin percentage) in both men and women. A volume coefficient of 43 is proposed for both sexes.

The suggestion is made that more accurate and satisfactory deductions of the normality or abnormality of erythrocyte numbers, hemoglobin, cell volume and the cell indexes of individual patients would result if each institution made use of standards found to be normal by its own peculiar technic.

We wish to express our thanks to Rev. Joseph M. Dougherty of Villa Nova College, whose interest and cooperation supplied us with the male subjects.

REFERENCES

(1) DILL, D. B.: Quoted by Walters.

(2) EMERSON, C. P.: Clinical diagnosis. Philadelphia: J. B. Lippincott Co., p. 484, 1921.

(3) HARROP, G. A.: The oxygen and carbon dioxide content of arterial and of venous blood in normal individuals, and in patients with anemia and heart disease. Jour. Exper. Med., **30:** 241–257. 1919.

(4) OSGOOD, E. E.: Hemoglobin, color index, saturation index, and volume index standards. Arch. Int. Med., **37:** 685–706. 1926.

(5) WALTERS, O. S.: Normal erythrocyte, hemoglobin and packed cell volume standards in normal men. Jour. Lab. and Clin. Med., **19:** 851–864. 1934.

(6) WINTROBE, M. M.: Blood of normal men and women. Bull. Johns Hopkins Hosp., **53:** 118–130. 1933.

(7) WINTROBE, M. M., AND MILLER, M. W.: Normal blood determinations in the south. Arch. Int. Med., **43:** 96–113. 1929.

THE USE OF CALCULATION CHARTS FOR THE WINTROBE HEMATOCRIT AND SEDIMENTATION RATES

F. LOWELL DUNN AND JOHN C. SHARPE

From the Department of Physiology and Pharmacology, University of Nebraska, College of Medicine, Omaha, Nebraska

The information derived from the hematocrit is a valuable addition in the clinical study of blood diseases. In order to gain its deserved popularity and to serve the clinician as a practical laboratory procedure, it must be accurate, simple, and not too time-consuming. Our experience with the Wintrobe[2,4] type of hematocrit fulfils these requirements in most respects. However we have found that the necessary calculations, though simple enough, are often a source of perplexity and error. This difficulty is especially pronounced with the occasional determination when there is a tendency to set the data aside for later calculation.

The extensive use of nomagraphic alignment charts for the simplification and speeding-up of calculations prompted us to construct such charts; (a) to correct the volume of packed erythrocytes for the amount of shrinkage that results from the use of an anticoagulant, (b) to calculate the mean corpuscular volume (C.V.) of the erythrocytes, (c) to calculate the mean corpuscular hemoglobin content (C.H.) of the erythrocytes and, (d) to calculate the mean corpuscular hemoglobin concentration (C.C.).

The method of making such charts has been fully described by Lipka.[1] Figure 1 gives directly the corrected packing of erythrocytes when 10 mgm. of dry potassium oxalate is used per 5 cc. of whole blood. Wintrobe corrected for this shrinkage by multiplying the volume of packed erythrocytes by the factor 1.09. If the ammonium and potassium oxalate mixture is used as recommended by Wintrobe then there is no shrinkage and this chart is not used. The amount of ammonia present

in this mixture however is sufficient to interfere seriously with nitrogenous determinations. We find that it is often convenient to collect blood for hematocrit studies and for blood chemistry at the same time. For general clinical work the use of potassium oxalate is preferable.

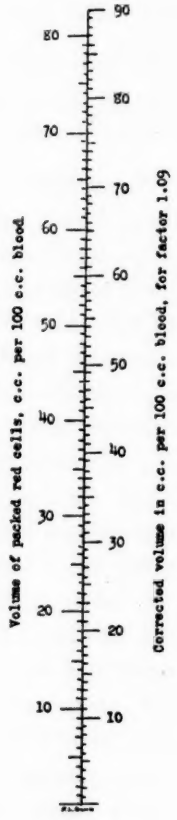


FIG. 1

Figure 2 is the chart for calculating the C.V., C.H., and C.C. This chart is made from the Wintrobe[3] formulas:

$$\text{C.V.} = \frac{\text{Volume of packed erythrocytes (cc. per 1000 cc. blood)}}{\text{Erythrocytes (in millions per c. millimeter)}}$$

Normal: 82–92 cubic microns

$$\text{C.H.} = \frac{\text{Hemoglobin (in gm. per 1000 cc. blood)}}{\text{Erythrocytes (in millions per c. millimeter)}}$$

Normal: 28–32 micromicrograms

$$\text{C.C.} = \frac{\text{Hemoglobin (in gm. per 100 cc. blood)}}{\text{Volume of packed erythrocytes (cc. per 100 cc. blood)}}$$

Normal: 32–26 per cent

The directions for use are as follows: A straight edge can be used but most satisfactory is a straight line scratched on a strip of clear x-ray film. This can be done with a needle. If the line is too fine it can be filled with ink or a glass marking pencil rubbing off the pigment that does not adhere to the scratch.

In figure 2 the middle column is the numerator, the column on the right the denominator, and the one on the left the quotient. Thus for the C.V. or the C.H. the straight line intersects the volume of packed cells or the hemoglobin on the middle column and the erythrocyte count on the right column. The result is read on the left column being expressed in cubic micra for the C.V. and in micromicrograms for the C.H.

For the C.C. the result is expressed in per cent. The hemoglobin value used on the chart is in grams per 100 cc. of blood and the packing in cubic centimeters per 10 cc. of blood. The result is then in per cent.

Our experience with the charts has been very satisfactory. They are simple to use, more rapid, and with proper care, extremely accurate. However, it should be pointed out that in using such charts, it is absolutely necessary to have a straight-edged rule. Any wrinkling or warping of the charts naturally introduces errors.

SUMMARY

1. The Wintrobe hematocrit is a valuable aid in the study of anemia.

2. We recommend the use of alignment charts for simplifying the calculations of hematocrit observations.

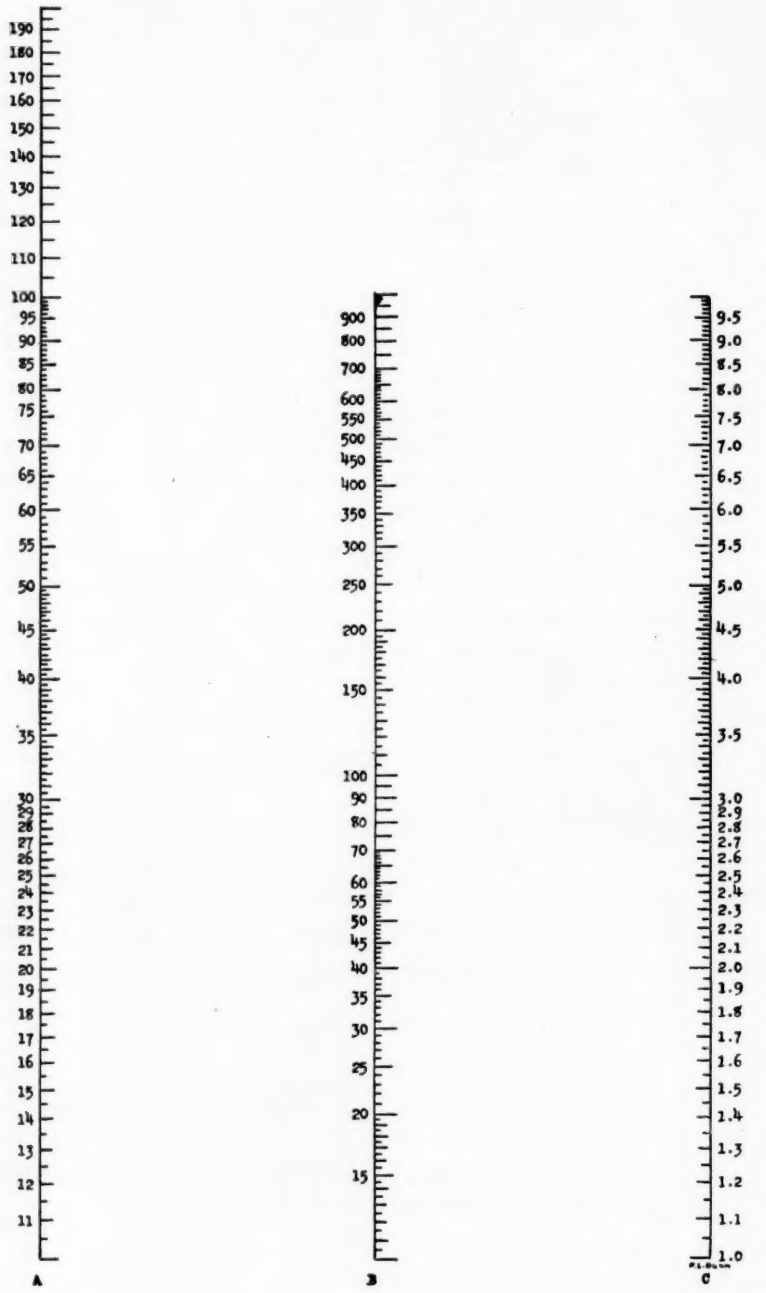

190
180
170
160
150
140
130
120
110
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
A
900
800
700
600
550
500
450
400
350
300
250
200
150
100
90
80
70
60
55
50
45
40
35
30
25
20
15
B
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.9
2.8
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
C

FIG. 2

REFERENCES

(1) LIPKA, J.: Graphical and mechanical computation. New York: John Wiley and Sons, **3:** 44–64. 1918.

(2) WINTROBE, M. M.: A simple and accurate hematocrit. Jour. Lab. and Clin. Med., **15:** 287–289. 1929.

(3) WINTROBE, M. M.: The size and hemoglobin content of the erythrocyte. Jour. Lab. and Clin. Med., **17:** 899–912. 1932.

(4) WINTROBE, M. M.: Macroscopic examination of the blood. Am. Jour. Med. Sci., **185:** 58–71. 1933.

A SIMPLE SLIDE TEST FOR SYPHILIS*

SEABORN J. LEWIS

Beaumont, Texas

The Wassermann reaction and several of the precipitation methods for syphilis have reached a very high degree of accuracy. I have, therefore, long hesitated to publish my results with a modification of the precipitation reaction. Having performed serological tests with some 27,000 serums, I feel it desirable to present the technic. Over 11,000 serums were tested by the Noguchi[8] method, 2,400 by the Wassermann (a modified Kolmer),[5] approximately 10,000 by the Kolmer, Kahn,[4] and "Lewis," 1,500 by the Kolmer, Kahn, Kline,[6] and "Lewis," and about 1,500 by the "Lewis" alone. These 1,500 serums were from prospective transfusion donors where rapid results were necessary. Many of these serums were run several times with several trial and error methods. In addition, a great many hundreds of serums were run with Butler at Tulane University, using various Wassermann and precipitation methods. In 1926, Butler[1] published his slide precipitation test for syphilis, and in 1929 another article of his[2] appeared, based on the Meinicke.[7] Later Johns,[3] published a modification of the slide test method, and Venable[9] also reported on such tests.

The chief difficulty in perfecting a satisfactory rapid slide test for syphilis has been in producing a uniform and stable antigen. By manipulation of the antigen constituents, an antigen can be had that is of a standard, low, or high degree of sensitivity and specificity. But constant duplication of an antigen of the proper degree of sensitivity has been a problem. The following procedures and technic were found to be satisfactory, and to give reliable results:

* Read before the Fifteenth Annual Convention of the American Society of Clinical Pathologists, held at Kansas City, Missouri, June 8 to 10, 1936.

METHOD

Stock antigen preparation

100 cc. of beef heart extract prepared exactly according to Kahn.
1.5 grams of powdered balsam of tolu.
0.4 gram of cholesterol (Pfanstiehl).

After adding the tolu to stoppered container shake vigorously for a few minutes and place in the incubator. Repeat the shaking twice a day for two days. Add the cholesterol and shake for a few minutes, place in the incubator for two days, and shake again twice a day. Filter and store in a glass stoppered bottle, or in one with a tin foil covered cork. The stock antigen, kept in the incubator or at room temperature in warm climates, remains stable for months. Protect it from the light.

The balsam of tolu is dried in the incubator until it is crisp or brittle, then placed between several thicknesses of brown wrapping paper, and hammered until pulverized. The coarse particles are discarded by using a hand sifter.

Saline benzoic acid diluent

100 cc. distilled water.
3 grams sodium chloride, c.p. (Baker's).
2 cc. of a one per cent alcoholic solution of benzoic acid.

Technic for performing the test

Preparation of antigen mixture: Into a small test tube measure 0.1 cc. of stock antigen and add 0.3 cc. of the saline diluent. At once shake the tube for a moment, and mix thoroughly for a few seconds by means of a capillary pipet with a rubber bulb, or a medicine dropper. Allow the mixture to stand for five minutes, and agitate again just before using. Use the antigen mixture within thirty minutes of its preparation.

Two drops of clear serum that have been heated at 56°C. for thirty minutes, are placed on a slide, and adjacent to the serum three drops (an equal quantity) of the antigen mixture. Mix with the broader flat end of a toothpick so that the mixture occupies the middle two thirds of the slide, and is not near enough to the sides to run over. The stirring should result in an oval spread with smooth margins so as to facilitate a rotary and side to side motion, and to prevent running over. Rock the slide from side to side for three minutes, and read within five minutes. Read by indirect light against a dark background, or by holding in front of, and just above a daylight bulb. A board painted black and with thin partitions to hold ten slides is convenient for running a number of serums. Ten is about the maximum number that can be properly mixed and agitated at one time. The test should not be performed in a cold room, as this causes a tendency towards false positives. Fans and strong drafts should be avoided.

Interpretation: Negative serums have a smooth, cloudy, opalescent appearance. Positive serums show various degrees of a brownish granular precipitate,

easily seen. Very strongly positive serums will show large clumps floating in an almost clear fluid. A minute amount or degree of precipitation should be disregarded or checked by other methods.

EXPERIMENTAL NOTES

Many combinations and variations of the constituents of the antigen described have been tried. The following conclusions are drawn:

(a) Two parts of diluent to one part of stock antigen, or four parts of diluent to one part of stock antigen give about the same results as the three to one proportion advocated above.

(b) Substituting normal saline and benzoic acid gave poor results.

(c) 3 per cent saline without benzoic acid, using heated serum, gives about equal results as with acid.

(d) 3 per cent saline without benzoic acid, using unheated serum, gives about equal results as with acid.

(e) Heated serum with benzoic acid gives clearer cut, more sensitive and specific results than any other combination.

(f) The factor of the pH is ignored in this technic, though sensitivity may be increased by adding hydrochloric acid, or decreased by adding sodium hydroxide.

(g) The presence of citrate in serum was found to be undesirable, rendering positive serums almost negative in reaction. This is important, as often one has at hand only citrated blood obtained for hematologic, chemical, or cultural purposes.

(h) Kahn and Kolmer antigens used according to the author's technic were unsatisfactory.

(i) Kahn and Kolmer antigens used according to Kline's technic were unsatisfactory.

(j) The author's antigen mixture may be substituted for the Kline antigen mixture and used as in his technic with good results.

(k) 1.25 per cent to 1.5 per cent balsam of tolu appeared to be the optimum amounts. Less tolu leads to too sensitive results, while greater amounts tend to obscure the reaction.

(l) Cold temperatures increase the antigen sensitivity, and warm temperatures decrease it.

Stock antigen adjustment

The above formula usually gives reliable results, but this antigen, as is true of all antigens, may require adjustment. Every new lot of antigen should be tested with known negative and all degrees of known positive serums from individuals with treated and untreated syphilis in all stages.

If the above formula results in antigen that is too sensitive, desensitizing reagent should be used. This is beef heart extract containing 1.5 per cent balsam of tolu. One part of desensitizing reagent should be added to nine parts of the new stock antigen. This mixture must be tried out with known

negative and positive serums, and if necessary smaller or greater proportions of desensitizing reagent should be added to the new antigen until the latter is of standard sensitivity. The entire new lot can then be corrected.

Antigen, less sensitive than standard can be corrected in like manner by adding sensitizing reagent to the new lot of antigen. The sensitizing reagent consists of beef heart extract containing 0.55 per cent cholesterol and 1.5 per cent balsam of tolu.

DISCUSSION

The antigen prepared and used as above has given results equal to the Wassermann, or any of the standard precipitation procedures. No attempt is made at this time to give figures or comparative results. In no instance has this test been strongly positive when the Kolmer, Kahn, and Kline were negative. In treated syphilis all of the standard precipitation technics and mine have proved somewhat more sensitive than the Wassermann. Extremely rarely has this technic given a negative or a moderately positive result that did not agree with the majority of three standard methods. The test has been used primarily to rule out syphilis, and where occasionally a weakly positive result has been obtained with this test, the result has been disregarded as evidence of syphilis unless the standard methods checked.

Most diagnostic laboratories employ, as I do, several methods in the serological diagnosis of syphilis. But the technic described is useful in ruling out the presence of syphilis, or in conjunction with one or more other methods.

SUMMARY

A simple, reliable slide test for syphilis is described. The results with thousands of serums have compared most favorably with several of the recognized methods used in the diagnosis of syphilis. The test is a modification of, and is based upon, the work of others who have utilized the precipitation principle in in the serology of syphilis.

REFERENCES

(1) BUTLER, H. W.: A simple, rapid precipitin test for the diagnosis of syphilis. New Orleans Med. and Surg. Jour., **79:** 105–106. 1926.

(2) BUTLER, H. W.: The adaptation of standard antigens to the precipitin test for syphilis, with comparative results shown by three thousand sera. Jour. Lab. and Clin. Med., **14:** 336–341. 1929.

(3) JOHNS, F. M.: Another modification of the precipitin test for syphilis. New Orleans Med. and Surg. Jour., **83:** 279–282. 1930.

(4) KAHN, R. L.: The Kahn test. Baltimore: Williams & Wilkins Co., 1928, pp. 201.

(5) KOLMER, J. A.: Studies in the standardization of the Wassermann reaction. Am. Jour. Syphilis, **6:** 1–32. 1922.

(6) KOLMER, J. A. AND BOERNER, FRED: Approved laboratory technic. New York: D. Appleton & Co., 1931, pp. 663.

(7) MEINICKE, ERNST: Ueber eine neue Methode der serologischen Luesdiagnose. Berl. klin. Wchnschr., **54:** 613–614. 1917.

(8) STITT, E. R.: Practical bacteriology, blood work and animal parasitology. Philadelphia: P. Blakiston's Son & Co., 1927, pp. 837.

(9) VENABLE, D. R.: The Butler-Meinicke reaction. Texas State Jour. Med., **28:** 409–412. 1932.

EDITORIAL

The Bone Marrow and Blood Dyscrasias

While the importance of the bone marrow as a hematopoietic center has long been appreciated, it is only recently that methods have been developed which enable its study in relation to blood dyscrasias, particularly those affecting the leukocytes.

These methods are so relatively simple, and their results of such apparent significance, that some summary of the reports concerning their use seems both timely and of practical interest.

The many and marked advances in hematology have made evident, among other things, that important as are the cytological studies of the blood in diseases of the blood, the blood picture thus obtained is nevertheless not always accurately indicative of the underlying abnormalities in the blood-forming organs. For it is, of course, readily apparent that the variations encountered in the blood picture are dependent, not only upon the phenomena attendant upon growth and destruction, but also upon the varied factors which govern the maturation and release of the blood cells from the hematopoietic centers.

That the existence, nature, and degree of such influences upon hematopoietic centers such as the bone marrow are far from obvious from a study of the circulating blood, has been shown by various observers. Thus, Damashek[2] has shown that in both pernicious anemia and primary hypochromic anemia, conditions in which the blood picture suggests bone marrow hypoplasia, the bone marrow may nevertheless be definitely hyperplastic; while Fitzhugh and Krumbhaar[5] have demonstrated a "pyoid" bone marrow in association with marked leukopenia.

It is, of course, recognized that the peripheral blood picture represents the end result of varied and often complex factors. But in view of the fact that identical pathologic processes may be

accompanied by diversified blood pictures and, further, that identical blood pictures may be encountered in fundamentally different blood dyscrasias, the necessity for study of what lies "behind" the blood picture becomes more and more apparent. And, as the bone marrow is one of the most important of the hematopoietic centers, the developments of methods for its clinical study become of immediate importance.

It must be said, however, that neither the idea nor its application is new, for clinical studies of the bone marrow were first recorded in 1908 (Ghedini) and can be found in the literature at intervals thereafter. The clinical study of the sternal bone marrow, however, originates from the work of Seyfarth in 1923 whose studies have since been continued and amplified by numerous investigators.

Two of the more recent methods for securing specimens from the sternal bone marrow are those described by Damashek[3] and Young and Osgood[9].

Damashek utilizes a modified Seyfarth trephine which is essentially a small steel trephine with a saw-toothed crown 5 mm. in diameter and 5 mm. long to the beveled edge.* The procedure is carried out under aseptic precautions (sterile gowns and rubber gloves).

The skin over the sternum in the region of the third and fourth interspaces is infiltrated with 1 per cent novocain, a 2 to 4 cm. incision made through the skin over the midline, and the periosteum, after being infiltrated with novocain, is incised and retracted with a small periosteal elevator.

The trephine is then placed directly over the bare surface of the bone and the bone marrow cavity entered by means of slight pressure combined with a rotating motion from side to side. A slight "give" indicates entrance when the trephine, with its enclosed plug of bone, is removed by a slight rocking motion. The cavity is then scooped out with a fine ophthalmologic surgical curette and the soft marrow thus obtained is spread on sterile glass slides while touch or imprint preparations are made from the bone plug or

* This instrument is obtainable from Codman and Shurtleff of Boston.

from bits of bone removed by the curette. The wound is then closed with one deep silk-worm gut suture and two to four horsehair skin sutures.

The procedure described by Young and Osgood is somewhat simpler.

With the patient lying on his back and the chest elevated by a pillow beneath the shoulders, the skin is prepared with iodine and alcohol. The sternomanubrial junction is located and the skin, subcutaneous tissues, and periosteum infiltrated with procaine hydrochloride.

The sternomanubrial junction is then entered in the midline at an angle of about 60 degrees to the surface of the chest with an 18 gauge spinal puncture needle 3 to 4 cm. long.

If much resistance is encountered penetration is facilitated by rotating the needle while maintaining pressure.

The stylet is then removed, a 2 cc. Luer syringe attached, and the marrow removed by aspiration. If aspiration is not successful, the stylet is replaced and the needle inserted a little deeper.

The aspirated material, which resembles blood, is transferred to a small test tube (4 x $\frac{1}{2}$ inch) containing a pinch of powdered potassium oxalate and shaken to ensure thorough mixing. Smear preparations may then be prepared at leisure.

The needle is withdrawn after replacing the stylet and the wound sealed with collodion.

With the method of Young and Osgood only smear preparations are possible. With Dameshek's method, in addition to smears, sections are also prepared from the bone plug removed by the trephine which is fixed in Zenker's solution containing 5 cc. of glacial acetic acid.

Decalcification is usually sufficient within 48 hours to permit sectioning; if not, further decalcification is necessary. Sections are stained with eosin-methylene blue.

While marrow smears are preferable for exact cytologic study, sections are valuable in the study of bone marrow relations and also of normal or abnormal hematopoietic islands.

In the study of bone marrow preparations the following cell types should be observed:[2]

- Nucleated erythrocytes
  - Normoblasts
  - Erythroblasts
  - Pro-erythroblasts
  - Megaloblasts
  - Promegaloblasts } in pernicious anemia
  - Erythrogones
- Leukocytes
  - Mature neutrophiles
  - Metamyelocytes
  - Myelocytes
  - Premyelocytes
  - Myeloblasts
  - Eosinophiles
  - Histiocytes
- Megakaryocytes.

Krumbhaar and Custer[7] call attention to the fact that differential cell counts of bone marrow are not simple procedures, especially in the study of sectioned material, and that while counts of well prepared smears are comparatively easy for the experienced worker, smear or imprint preparations are inaccurate in the study of the relative proportions of various cell series because closely packed cell clumps do not tend to disperse or may even adhere to narrow interstices and thus not be included on the slides. They believe, therefore, that the truest pictures of the status of hematopoietic tissue are obtained from the study of sections.

They prefer a magnification of 1500 diameters (15 x ocular and 1.2 mm. oil immersion objective), an ocular micrometer being essential.

In their opinion a count of 500 cells suffices except when the incidence of sparsely and irregularly distributed cells is an issue, in which case the incidence of such cells in 200 fields is determined.

Damashek[2] from a study of 200 bone marrow sections obtained at necropsy, presents the following as representing the approximate cellular constituents found in normal (rib) marrow: ratio of nucleated erythrocytes to leukocytes: approximately 1:1 (variation 0.6 to 1: 1.2 to 1).

Differential leukocyte count: mature neutrophiles, 2 to 5 per cent
metamyelocytes, 20 to 40 per cent

Differential leukocyte count:	myelocytes, 30 to 50 per cent
	premyelocytes and myeloblasts, 1 to 5 per cent
	eosinophiles, 1 to 5 per cent
	histiocytes, 1 to 2 per cent
Differential erythrocyte count:	normoblast, 80 per cent
	erythroblast, 20 per cent

From two to ten megakaryocytes are usually seen while counting 300 leukocytes (0.7 to 3.3 per cent).

Damashek has reported bone marrow studies in a variety of "blood diseases" and comments that anemia, leukopenia, and thrombocytopenia form a triad pointing to a "destructive" ("myelopthisic") process in the bone marrow which is usually leukemic in type.

In his series bone marrow studies demonstrated that many cases of so-called Banti's disease, splenic anemia, agranulocytosis, aplastic anemia, and even pernicious anemia were in reality instances of aleukemic leukosis.

The diagnostic value of bone marrow studies is thus readily apparent.

Bone marrow studies in two cases of infectious mononucleosis are reported upon by Freeman[6] who found the marrow spaces in both cases packed with mature and immature lymphocytes, suggesting that glandular fever may really be an abortive, benign form of acute lymphatic leukemia.

The bone marrow findings in agranulocytosis are reported by Custer[1] to be (in nine of eleven cases):

a) Marked proliferation of myeloblasts; b) failure of these cells to mature, resulting in paucity of myelocytes and practically complete absence of segmented forms; c) normal or slightly increased erythrocyte formation; d) slight hyperplasia of otherwise normal megakaryocytes; and e) infiltration of lymphocytes and plasmocytes.

The same observer, in connection with the utilization of bone marrow studies in the diagnosis of blood dyscrasias, points out that because of the wide distribution of bone marrow and its variability in different bones at different ages:

1) Examinations based on marrow from but one locus may be inadequate; 2) the tibial and femoral shafts may be taken as representative of normally fatty marrow in the adult and the ribs, sternum, and vertebrae as examples of normally cellular marrow in the adult; 3) in any case the tibia and femur as well as the sternum are preferably examined; 4) that sections must be of a character enabling accurate cell identification and 5) that smears alone are of much less value than the results of examinations of both smears and sections.

The changes encountered in the bone marrow in agranulocytosis have also been reported upon by Darling, Parker, and Jackson.[4]

From a study of twenty-five cases they conclude that the uniformity of the pathological changes in the bone marrow suggests that agranulocytosis is probably a disease entity.

In their experience the salient features of the bone marrow in rapidly fatal cases were lack of maturation in the granular series and hyperplasia of the stem cells. Cases of longer duration presented hypoplasia of the myeloid tissue with the coincident appearance of numerous lymphocytes and plasma cells. In none of their cases was there any evidence of obvious changes in the erythrocyte series or of degenerative changes in the white cell series.

Recovery is characterized by a rapid development of stem cells into myelocytes and polymorphonuclear neutrophiles, this sequence of events tending to substantiate the concept of "maturation arrest" advanced by Fitz-Hugh and Krumbhaar, and also by Custer. As Fitz-Hugh and Krumbhaar have pointed out, "maturation arrest" seems more suitable as a descriptive term, in view of the presence of many myeloblasts in the bone marrow, than granulocytic aplasia. The same observers also suggest "pernicious leukopenia" as a name for this disease because of the absolute reduction of both lymphocytes and neutrophiles as well as because of certain other analogies to pernicious anemia.

The bone marrow in idiopathic thrombopenic purpura has been reported upon by Lawrence and Knutti[8] who conclude from a study of six cases, in four of which no bone marrow changes were found, that there may be two types of this disease, one in which

bone marrow changes are absent, and one characterized by a decrease in megakaryocytes as described in the few available reports of bone marrow studies in this condition.

In any event, it appears that future studies of blood dyscrasias may well be fortified by studies of bone marrow preparations as well as studies of the peripheral blood, and that from such correlated observations the future understanding of many blood dyscrasias at present but little understood and now regarded as of uncertain origin may well develop.

REFERENCES

(1) Custer, R. P.: Studies on the structure and function of bone marrow. IV. Bone marrow in agranulocytosis. Am. Jour. Med. Sci., **189:** 507–515. 1935.

(2) Dameshek, W.: Primary hypochromic anemia (erythro-normoblastic anemia): A new type of idiopathic anemia. Am. Jour. Med. Sci., **182:** 520–553. 1931.

(3) Dameshek, W.: Biopsy of the sternal bone marrow. Am. Jour. Med. Sci., **190:** 617–640. 1935.

(4) Darling, R. C., Parker, F., Jr., and Jackson, H., Jr.: The pathological changes in the bone marrow in agranulocytosis. Am. Jour. Path., **12:** 1–11. 1936.

(5) Fitzhugh, T., Jr., and Krumbhaar, E. B.: Myeloid cell hyperplasia of bone marrow in agranulocytic angina. Am. Jour. Med. Sci., **183:** 104–110. 1932.

(6) Freeman, W.: Bone marrow studies in glandular fever. Am. Jour. Clin. Path., **6:** 2–185. 1936.

(7) Krumbhaar, E. B., and Custer, R. P.: A note on differential cell counts of bone marrow. Am. Jour. Med. Sci., **189:** 630–633. 1935.

(8) Lawrence, J. S., and Knutti, R. E.: The bone marrow in idiopathic thrombopenic purpura. Am. Jour. Med. Sci., **188:** 37–41. 1934.

(9) Young, R. H., and Osgood, E. E.: Sternal marrow aspirated during life. Arch. Int. Med., **55:** 186–203. 1935.

—R. A. Kilduffe

NEWS AND NOTICES

The American Board of Pathology

A bulletin containing information concerning the American Board of Pathology is now available and may be obtained from Dr. Frank Hartman, Secretary, Henry Ford Hospital, Detroit, Michigan. In it one will find the requirements which are as follows:

A. General qualifications.
   1. Satisfactory moral and ethical standing in the profession.
   2. License to practice medicine.
   3. Membership or associate membership in the American Medical Association or by courtesy membership in such Canadian or other national medical societies as are approved by the Council on Medical Education and Hospitals of the American Medical Association.
   4. That the applicant devotes his time primarily and principally to the practice of pathology.

B. Professional education.
   1. Graduation from a medical school in the United States or Canada, approved by the Council on Medical Education and Hospitals of the American Medical Association.

C. Special training, to be effective after July 1, 1938:
   1. Completion of an interneship of not less than one year in a hospital approved by the Council on Medical Education and Hospitals of the American Medical Association.
   2. A period of study, exclusive of interneship, of not less than three calendar years, exclusive of reasonable vacation periods, in an institution or pathologic department recognized by the same Council and the Board of Trustees as competent to provide a satisfactory training in the field of pathology. This period of special training preparation shall include the following:
      (a) Graduate training for one year in the various phases of clinical pathology.
      (b) Training and experience of not less than two years in a department of pathologic anatomy.
      (c) Such training may be combined or in sequence.

D. Special qualifications.

1. The Board may accept candidates without special training as outlined in Section C above provided that:

(a) The candidate shall have been for a period of five years of professorial rank in a department of pathology in an approved medical school.

(b) The candidate shall have been practicing pathology for ten years in a class A hospital, having an adequate department of pathology, and approved by the Council on Medical Education and Hospitals of the American Medical Association.

Candidates with special qualifications, as outlined in Section D above, may be certified without examination, at the discretion of the Board, up to July 1, 1938.

Certification is of two types: (1) pathological anatomy, and (2) clinical pathology. The examination fee is $35.00. If a sufficient number make application, the first examination will be held in Baltimore at the time of the meeting of the Southern Medical Association.

It will be apparent that at least one matter will have to be further clarified since under these rules no provision is made for certification of a candidate from a medical school of any other country except the United States and Canada. Furthermore, it will be desirable to make clear the meaning of the special qualifications; that is, whether the candidate must have completed (a) or (b) or (a) and (b).

The American Board of Internal Medicine has announced its rules for certification, copies of which may be obtained from Dr. Walter L. Bierring, Chairman, 406 Sixth Avenue, Des Moines, Iowa.

BOOK REVIEWS

Syphilis sine Morbus Humanus. By Charles S. Butler. Brooklyn, New York, pp. xiv + 137. 1936.

This pithy and very enjoyable little treatise was written "for scientists and laymen interested in the damage to men from venereal diseases." It was written (1) to give popular knowledge, (2) to expose the fallacy of the American-origin-of-syphilis hypothesis, and (3) to show the unity of so-called "yaws" and syphilis. Admiral Butler has brought together some of the most interesting of the older medical writings and has digressed from his subject to give personal recollections and first-hand information of certain tropical countries in which he has served as medical officer of the United States Navy. His arguments, flavored with salt and directly to the point, will stimulate any reader and cause his "adversaries" many uneasy moments.

For effective, profitable, and interesting medical reading the book is recommended to pathologists and others who seriously study syphilis.

The Students Manual of Microscopic Technique. By J. Carroll Tobias. Boston: American Photographic Publishing Co., pp. xviii + 210, 1936. $2.50.

This book deals with elementary principles of microscopic technic with special reference to specimens from animals and plants, but without much specific reference to human material. The book seems to be designed for that great and increasing field of amateur microscopists. The last chapter is devoted to photomicrography and in it one will find useful and clearly described methods.

The Patient and the Weather, vol. 1, part 2: Autonomic Integration. By William F. Petersen. Ann Arbor, Michigan: Edward Brothers, Inc., pp. xxxii + 781, 1936. $9.00.

This huge volume contains further data which the author believes support his conclusions that the weather plays a profound rôle in medicine and that it greatly affects the patient. There are 358 figures, many tables and references to literature. The author's general conclusion in his own words is: "The organism adjusts to the meteorological environment by the three major components of the autonomic apparatus—chemical, endocrine, nervous." Petersen believes these changes have rhythm and that the swings are of clinical significance.

Pathology of the Nervous System. By J. Herny Biggart. Baltimore, William Wood & Co., pp. xvi + 335, 1936. $5.25.

The author, while recognizing general pathology as the parent, nevertheless believes neuropathology has grown up enough to justify its right to intensive treatment in a separate course. The book is intended for students, especially those majoring in neurology and psychiatry. The first chapters deal with the general reactions of the nervous tissue and with changes in the cerebrospinal fluid. The following chapters are on vascular disease of the brain, acute bacterial infections, chronic infections, diseases due to virus, those of unknown etiology, intoxications and deficiency diseases, degenerative diseases, injuries to the nervous system, tumors and developmental errors.

The book is illustrated with 204 photographs and photomicrographs, all excellently selected and of fine quality. The style of exposition is simple, clear and concise. Altogether it is an excellent text and modern in every respect. It can be recommended without reserve.

A Textbook of Pathology. By W. G. MacCallum. 6th Ed. Pp. xvi + 1277, 1936. Philadelphia, W. B. Saunders Company. $10.00.

This text by MacCallum is so generally wellknown that it is not necessary to review its chapters in detail. It should be emphasized, however, that in this edition MacCallum, unlike at least one of his colleagues, has found so much new in pathology in recent years as to make it necessary to completely revise this

edition. This he says was found more than ever necessary because of the extraordinary advances, especially in the field of endocrine disturbances, vitamine deficiencies, and virus infections. The text departs from the usual one in pathology in that it treats diseases from the etiologic factors and does not deal with pathology by organs. The references given at the end of chapters are in sufficient numbers for the average medical student but naturally would not satisfy a student specializing in pathology. It is hoped that the next edition will contain a modernization of the chapter dealing with animal parasites.

Tissue Immunity. By Reuben L. Kahn. Springfield, Illinois, Charles C. Thomas, pp. xx + 707, 1936. $7.50.

This monograph, perhaps the most outstanding treatise on immunity ever published, will be of great interest and importance to the pathologist, bacteriologist, physiologist, biologist, and clinician. It is not a summary of literature, although there are many references; it is the record of results of laboratory experiments performed by the author during the past five years. From these experiments Kahn has evolved his theories and conclusions concerning the nature of immunity and then examined the experience of clinicians in an attempt to explain the observed phenomena. In this he has succeeded remarkably well, giving for the first time clear and logical explanations to many known experiences. He has discarded the expression "desensitization" in favor of "disimmunization" and fortifies his decision by experimental results. His reasons why small doses of antigens are to be preferred to large ones, why vaccines should be given not more frequently than every week, why serums are effective when given intravenously, why subcutaneously administered vaccines for furunculosis are ineffective, why children can withstand more biological therapy than adults, why they are more susceptible to certain diseases and less so to others, why an adult becomes immune to diphtheria and scarlet fever, why a smallpox vaccination necrotizes and many other similar questions are given ingenious and vital explanations. His demonstration that tissue cells have the important rôle in immunity is exhaustive and conclusive.

In the book Kahn thinks aloud, hence at times the writing is a bit verbose, however, the scanner will find a brief summary at the end of each chapter and finally terminal chapters where the meat of the book will be found. The protocols are appended at the end of each chapter. Some improvement could frequently be made on sentence construction and terminology of organisms, *Spirochetae pallida* (sic!) being used throughout the text, but on the whole there is no mistaking the meaning of his sentences, they being for the most part in conversational tone.

Prophesying in science is dangerous and rash, however, the reviewer is willing to risk the opinion that this book will not be long in becoming a classic in fundamental medicine and will stimulate more productive investigation in immunology than any previous contribution.

Chemical Procedures for Clinical Laboratories. By Marjorie R. Mattice. Philadelphia: Lea & Febiger, pp. 520, 1936. $6.50.

This book, the outcome of a laboratory course in the New York Post-Graduate Medical School of Columbia University, is primarily designed for the analyst. It is a compilation of methods of chemical procedures, most of which may be considered thoroughly standardized. The book is divided into sections consisting of chemical analysis of the blood, urine, gastro-intestinal secretions, cerebrospinal fluid, effusions and other body fluids. The appendix contains a resumé of normal data, various technical notes in regard to reagents and various tables. The methods given are clearly described, reference to the original description being given, and the author has included numerous tables for simplifying calculations. The book can be heartily recommended.

Classification of Yeasts and Yeast-like Fungi. By C. Virginia Fisher and Lloyd Arnold. Urbana: University of Illinois Bulletin, Illinois Medical and Dental Monographs, vol. 33, no. 51, pp. 92, 1936. $1.00.

Clinical pathologists will welcome this excellent study of a most confused subject, the authors having clarified the issues

considerably. They conclude that on the basis of morphology alone, yeast-like fungi can be separated into Saccharomyces, Cryptococcus, Endomyces, and Monilia. Further studies on corn meal will usually result in the identification of species. The authors conclude that *Monilia albicans* is the only pathogenic species of monilia and furnish tables showing not only its incidence but that of other yeast-like fungi as well. Not the least valuable part of the work is the very complete list of bibliographic references.